Modulation of the humoral immune response by an FcγRII/III specific scFv in mice

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List of abbreviations:

ADCC: antibody-dependent cellular cytotoxicity

AEC: aminoethyl-carbazole

BCR: B-cell receptors

BirA: biotin-ligase

BLNK: B-cell linker protein

BSA: bovine serum albumin

Btk: Bruton's tyrosine kinase

CDR1-3: complementarity determining region 1-3

CTLA-4: cytotoxic T-lymphocyte-associated protein 4

DAG: diacyl-glycerol

DC: dendritic cell

DC-SIGN: dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

Dok-1: docking protein-1

ELISPOT: enzyme-linked immunospot assay

FcγRs: Fcγ-receptors

FDC: follicular dendritic cell

FITC: fluorescein-isothiocyanate

FITC-dextran: FITC conjugated to dextran

GC: germinal center

GPI: glycosylphosphatidyl inositol

Grb2: growth factor receptor associated binder 2

IC: immune complex

IL-: interleukin

ITAM: immunoreceptor tyrosine based activation motif

ITIM: immunoreceptor tyrosine based inhibitory motif

Itk: IL-2 inducible T-cell kinase

IP_3: inositol-1,4,5-trisphosphate

IPTG: isopropyl β-D-1-thiogalactopyranoside

KLH-FITC: FITC-conjugated keyhole limpet hemocyanine

Lck: leukocyte-specific protein tyrosine kinase

Lyn: v-yes-1 Yamaguchi sarcoma viral related oncogene homolog

Hck: haemopoietic cell kinase

LPS: lipopolysaccharide

MARCO: macrophage receptor with collagenous structure

MAPK: mitogen activated protein kinase

PI3K: phosphatidyl-inositol-3 kinase

PLCγ: phospholipase C γ

PRR: pattern recognition receptor

PTB-domain: phosphotyrosine-binding domain

RA: rheumatoid arthritis

ROI : reactive oxygen intermediates

scFv : single chain Fragment variable

SDS-PAGE : sodium-dodecyl-sulphate-polyacrylamid gelelectrophoresis

SHIP-1: SH2-domain containing polyinositol-5-phosphatase

SLE: systemic lupus erythematosus

SLP76: SH2 domain containing leukocyte protein of 76kDa

SLS: sodium- lauroylsarcosin

Syk: spleen tyrosine kinase

TD-response : T-dependent immune response

TI-2-response : T -independent type 2 immune response

TNFα : tumor necrosis factor α

ZAP70 : Zeta-chain-associated protein kinase 70

1. Introduction

Fc-receptors play a central role in the regulation of both the efferent and afferent phase of the immune response by setting the threshold of B cell activation, regulating DC maturation (1) and functioning as a bridge between innate and adaptive immunity (2). Due to the ability of immune complexes to bind all types of IgG Fc receptors via the conserved Fc-portion of IgG, activating type Fcγ-receptors (FcγR) may facilitate recruitment and activation of inflammatory cells and antigen presentation to effector cells, while the inhibitory FcγRIIb may transmit antagonistic signals with a possible outcome of inhibiting cell activation. Several previous *in vivo* studies aimed to examine the nature of this fine balance between positive and negative signals with yet controversial results (3) (4).

Affinities of FcγRs are variable, low affinity receptors, FcγRII and FcγRIII only bind multiple Fc in immune complexes, and are less restricted to the isotype of the antibody, whilst the high affinity FcγRI shows a strict preference towards binding monomeric or complexed IgG2a (2) (5).

Activating Fc-receptors share a common consensus immunoreceptor tyrosine based activation motif (ITAM) in the intracellular part of the γ-chain, which becomes phosphorylated upon ligand binding, thus forming docking sites for SH2-domain containing signalling proteins (6) (7). The only yet described inhibitory receptor of the Fc-receptor family is the single chain FcγRIIb, which is widely present on immune cells, though is the only classical Fc-receptor expressed on B cells (8). It contains an intracellular immunoreceptor tyrosine based inhibitory motif (ITIM), which becomes phosphorylated by Src family kinases upon co-ligation of FcγRIIb with activating receptors, such as B-cell receptors (BCR) via immune-complexes.

Several groups reported on the *in vivo* immuno-modulatory effects of immune-complexes. IgG1-, IgG2a- and IgG2b-containing complexes can mediate immune response with dual outcomes, depending on the type of the antigen. Based on its unique structure, IgG3 has the capacity to self-associate and activate the complement system, and enhance the immune response (9). As IgG-mediated suppression also occurs in mice lacking the known FcγRs, this process is most possibly Fc-receptor independent, and caused by epitope masking (10). On the other hand, FcγRIIb-deficient mice respond to immune-complex treatment with an up-to 100-fold elevation of the antibody titers (11). Takai et al. demonstrated that mice with FcγRIIb deletion are highly susceptible to a more severe form of collagen-induced

arthritis, whilst animals lacking the common γ-chain of the activating Fc-receptors including FcγRI and FcγRIII do not develop the disease at all (12) (13).

As the *in vivo* data about the regulatory function of immune complexes on the humoral immune response are controversial, we carried out a set of experiments where we aimed to collect more data about the localisation and *in vivo* effect of intravenously administered and well-characterised immune complexes. Several animal models of inflammatory diseases underline the importance of FcγR-function (14) (15, 16) therefore the further understanding of the complexity of FcγR-mediated signalling events could highly contribute to the development of efficient drugs diminishing inflammation in autoimmune diseases.

1.1 Structure of Fcγ-receptors

Fc receptors are members of the immunoglobulin superfamily (exception is the C-type lectin FcεRII) that bind the Fc part of the immunoglobulin (Ig) molecules, and function as non pre-determined receptors of the immune complex (IC). Based on their preference towards different Ig-isotypes, Fcα/μ–, Fcγ–, Fcδ– and Fcε–receptors have been identified. Depending on the structure, single-chain and multi-subunit receptors exist; and on the basis of the consensus motifs expressed in the cytoplasmic part, activating and inhibitory receptors are known. The affinity of the ligand binding also varies: high affinity $K_d=10^{-8}$M, and low affinity receptors contribute to the fine-tuning of the immune response. All of the identified classical and non-classical Fc-receptors are listed in Table 1 (17) (12).

Based on the structure, multi-subunit and single-chain Fcγ receptors exist (Figure 1.) (66) (67) (68). The multi-subunit members of the family (FcγRI and FcγRIII) are composed of an α chain, which is the subunit responsible for the ligand binding, and at least one signal-transducing γ chain dimer, closely related to the ζ chain of the T-cell receptor complex that contains a highly conserved consensus sequence, the immunoreceptor tyrosine based activation motif (ITAM). ITAMs contain two tyrosine residues separated by around 9–12 amino acids; the canonical ITAM sequence is YXX[L/V]X6–9YXX[L/V] (Y=tyrosine, L=leucine, V=valine, and X stands for any amino acid) (18).

In case of the single-chain receptor FcγRII the ligand recognition domain and the signal-transmitting sequence are encoded within the same chain, containing either an ITAM or an immuno-receptor tyrosine based inhibitory motif (ITIM, [I/V/L/S]XYX2[L/V]) (8), depending on the isoform: the human FcγRIIa is an activating receptor, expressed mainly on macrophages, neutrophils, eosinophils, platelets and Langerhans cells, while the human FcγRIIb1, the main isoform present on B cells and FcγRIIb2 are inhibitory type receptors. Due to an additional membrane-proximal sequence (which is caused by the translation of all 4 exons of the intracellular chain), the b1 isoform is not internalised, while b2 mediates phagocytosis in macrophages, neutrophils and eosinophils (65). In mice, 4 types of Fcγ receptors exist: FcγRI-IV, where FcγRII has inhibitory functions exclusively, FcγRI, III and IV have activating functions and FcγRIV resembles homology to the human FcγRIIIa isoform. Murine B cells exclusively express FcγRIIb1. (2)

While mouse FcγRI (K_d=10^{-8}M) displays high affinity towards IgG2a isotype specifically (it has a low affinity binding capacity for IgG3 as well), FcγRII and FcγRIII bind the IgG Fc region with a low affinity (K_a=10^5-10^6M), but a broader isotype-binding capacity (IgG1, IgG2a, and IgG2b). In mice, the most potent effector isotypes are IgG2a and IgG2b; involvement of members of the complement cascade in their effector mechanisms has been suggested though.

The recently identified FcγRIV is of intermediate affinity and restricted subclass specificity towards IgG2a and IgG2b (5, 19). The receptor is expressed on neutrophils, monocytes, macrophages, dendritic cells, mast cells and lymphocytes, and can be up-regulated upon inflammatory stimuli or T_H1 cytokines, while T_H2 cytokines or induction of cell maturation down-regulate cell surface expression of FcγRIV.

The pattern of Fcγ receptor expression varies on different effector cells so that the isotype of the antibody determines which accessory cells are involved in the immune response.

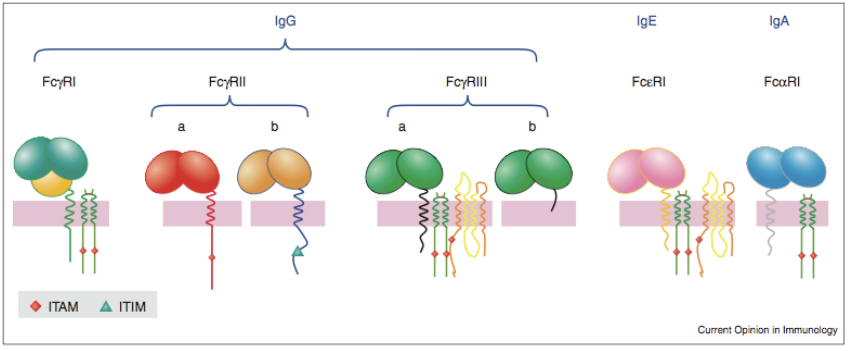


Figure 1.: Schematic structure of the human Fc-receptors (20)

Receptor	Cellular distribution	Expression in mouse/human	Function
FcγRI (CD64)	Mo, Ma, Neu, Eo, DC,	m/hu	ADCC, IC phagocytosis, oxidative burst, cytokine release
FcγRIIa (CD32A)	Mo, Ma, Eo, Neu, thrombocyte, DC, LC	hu	phagocytosis, oxidative burst
FcγRIIb (CD32B)	B-cell, mast cell, Ma, Ba, Eo, Neu, DC, LC	m/hu	negative regulation of the cellular response
FcγRIIIa (CD16A)	Ma, Mo, NK cell, mast cell, Eo, DC, LC, Neu (mouse)	m/hu	phagocytosis, ADCC
FcγRIIIb (CD16B)	Neu, Eo	hu	cell activation in co-aggregation with the FcγRIIa
FcγRIV	Neu, Mo, Ma, DC	m	phagocytosis
FcεRI	mast cell, Ba, Eo, DC, LC	m/hu	degranulation
FcεRII (CD23)	ubiquitous, platelet	m/hu	regulation of IgE production
FcαRI	Ma, Neu, Eo	hu	phagocytosis, production of free radicals, ADCC
FcRn	placenta, small intestine, Mo, DC	m/hu	foetal immunity, IgG turnover
Fcα/μR	B-cell, Ma	m/hu	not clear
Poly-IgR	epithelial cells, liver, small intestine, lungs	m/hu	secreted IgA, IgM transport
FcRH1-5	B-cell	m/hu	not known

Table 1.: The Fc receptors

Mo=monocyte, Ma=macrophage, Neu=neutrophil granulocyte, Ba=basophil granulocyte, Eo=eosinophil granulocyte, Dc=dendritic cell, NK=natural killer cell, LC=Langerhans-cell, IC=immune complex, ADCC=Antibody Dependent Cellular Cytotoxicity

1.2 The role of Fcγ-receptors in the regulation of the immune response

In the course of an infection, early responses are characterised by the activation of the innate immune system in order to avoid spreading of the pathogen: on many microbial surfaces (e.g. the ones poor in sialic acid), the spontaneously cleaved C3b is bound, and initiates the alternative way of complement activation. Surfaces rich in mannose-containing carbohydrates facilitate the mannan-binding lectin dependent complement activation pathway - capsular polysaccharides induce T-cell independent-2 responses, followed by the production of low affinity IgM antibodies, which in turn can initiate the classical way of complement system activation. Internalisation of the pathogen may be initiated by complement receptors expressed on phagocytotic cells.

Antigen recognition and uptake also occurs in innate cells via pattern recognition receptors (PRRs), like the mannose receptor or scavenger receptors on macrophages (e.g. MARCO or CD36), as well as by complement receptors, followed by phagocytosis, cytokine release and recruitment of accessory cells (21).

Fcγ receptors play an important role in the regulation of the immune response as they represent a link between the innate and adaptive immunity. In the early phase of the immune response, IgG3 is the first IgG class produced. The isotype switch is regulated by IFNγ originating from **NK cells** and Th1 cells, which induces up-regulation of FcγRI expression. In the course of an advanced T cell dependent immune response, as a result of Th1 activity, large amounts of affinity-matured IgG2a antibodies are produced (69), high affinity ligands for FcγRI. Ligation of FcγRI leads to antigen capturing, internalisation, and antigen presentation at least with a 10-fold greater intensity than in the case of the low affinity Fc-receptors (22).

Upon viral infection, the effected cells are usually recognised and destroyed by cytotoxic T cells due to viral peptides presented on their MHCI molecules. Viral proteins expressed on the cell surface can also be recognised by specific antibodies, so that NK cells can eliminate the antibody-coated infected target cells via antibody dependent cellular cytotoxicity (ADCC) (70). This process is mediated by the low affinity receptor FcγRIII (CD16), which binds IgG1 and IgG3, and the outcome is the release of cytoplasmic vesicles containing perforin and granzymes destroying the antibody coated cell.

The most important phagocytotic cells participating in the clearance of invading bacteria are **neutrophil granulocytes** and **macrophages**. These cells are capable to ingest and destroy many pathogens directly, however, ‘labelling’ the cell surface antigens of bacteria (e.g. the polysaccharide capsule) with antibodies and/or complement, a process called opsonisation

highly enhances the effectiveness of phagocytosis. Macrophages express a whole set of Fcγ-receptors on their surface (FcγRI, FcγRIIa, FcγRIIb and FcγRIII). The receptor-mediated antigen up-take is followed by lysosomal destruction, and the release of inflammatory cytokines like IL-1, IL-6 and TNFα in order to recruit other phagocytotic cells to the site of pathogen invasion (71).

Neutrophils represent the most abundant population of leucocytes in the circulation, and they are the earliest cell type mediating an inflammatory response towards invading pathogens. Besides the production of reactive oxygen intermediates (ROI), proteolytic enzymes and anti-microbial molecules, neutrophils participate in the phagocytosis of the pathogens as well. Several different receptors facilitate the antigen recognition: receptors for complement cleavage products and pathogen-associated molecular pattern recognition receptors, and Fc-receptors. Human neutrophils constitutively express FcγRIIa and glycosylphosphatidyl inositol (GPI)-anchored FcγRIIIb, while FcγRI-expression is inducible by IFNγ. FcγRIIIb lacks intracellular signalling domains, it only can trigger cell activation if associated with FcγRIIa. On mouse neutrophils though, the ITAM-containing FcγRIIIa and the ITIM-containing FcγRIIb are present (72).

Distribution of FcγRIIa and FcγRIIb on human **dendritic cells** (DC) also has a strong influence on regulating immunity and tolerance: signalling events mediated by the IIb isoform on DCs limit cell maturation and support induction of T cell tolerance, while the activation of monocyte derived DCs via FcγRIIa leads to secretion of a set of cytokines like IL-10, IL-6, TNF-α and IL-8 (1).

In the late phase of the humoral immune response, it is essential to down-regulate antibody production in order to prevent further tissue damage. When immune complexes cross-link the **B cell** receptor with the FcγRIIb, inhibition of cell activation occurs (73) (74) (75). (Molecular mechanisms of this process are described in section **1.5**.) Defects at this step can lead to autoimmunity due to impaired function of the inhibitory receptor.

1.3 The role of IgG-containing ICs in the regulation of the immune response

In order to maintain the fine balance within the immune system, and avoid excessive or ineffective responses towards an antigen, several regulatory mechanisms exist. One of these is the co-expression of receptors with similar ligand binding properties but different signalling outcomes on the same cell. Fcγ-receptors share common ligands, however, their preferences towards different isotypes vary and they also differ in the signal-transmitting intracellular sequence. Therefore, they represent an optimal choice for such a regulatory system. Immune complexes have been long described to have regulatory functions, mediating a wide spectrum of responses, from the induction of a complete inhibition of antibody production the enhancement of the humoral immune response.

The most excessively studied experimental setup to study the IC-mediated modulation of the immune response is the injection of erythrocytes as particulate antigens and erythrocyte-specific IgG in close temporal relationship, where the humoral immune response towards erythrocytes is suppressed: either due to the co-cross-linking of BCR and FcγRIIb; induction of enhanced phagocytosis and rapid clearance of erythrocytes from the blood by phagocytotic cells or masking of the antigen epitope on erythrocytes for B cell recognition (4) (76).

Experiments carried out in knock-out mice showed that – in response to erythrocytes as particulate antigens, - IgG-mediated suppression is intact by reduced levels of FcγRI expression, as well as in the absence of FcγRII and FcγRIII: it occurs most probably via epitope-masking (77).

On the other hand, administration of ICs composed by monoclonal antibodies and soluble antigens enhanced the antigen-specific humoral response, proving that the nature of the antigen (particulate vs. soluble) is important concerning the IC-mediated function. The IgG1-, IgG2a; and IgG2b-mediated enhancement of antibody synthesis is strongly reduced in γ-chain knock-out animals, while FcγRIIb-deficiency manifests in an overshoot of antibody production (78). In the later case, the suggested mechanism is the lack of co-cross-linking by ICs.

Several animal models of IC-mediated disorders, e.g. collagen-induced arthritis, haemolytic anaemia or systemic IgG- and IgE-induced anaphylaxis demonstrated that lack of FcγRIIb leads to robust effector cell responses and to induction of severe inflammation (3).

Depending on the isotype of the antibody, ICs can mediate maturation of DCs. Ligation of

FcγRIIa on immature monocyte-derived DCs induces maturation, increases antigen presentation to allogenic T cells, and enhances secretion of many inflammatory cytokines including IL-10, IL-6, TNFα and IL-8. Co-ligation of FcγRIIb limits this activation, while targeting the inhibitory receptor alone maintains an immature state (1, 5).

Inflammatory effector cells, like mast cells, macrophages and neutrophil granulocytes express both activating and inhibitory type Fcγ-receptors, thus the cellular response is strictly dependent on the isotype of the antibody in IC.

1.4 Possible involvement of Fcγ receptors in systemic autoimmunity

Systemic autoimmune diseases are induced by the development of an immune response against self-antigens e.g. nuclear proteins from apoptotic cell debris or structures present at an immune privileged site of the body. As the antigen cannot be eliminated completely, the response remains sustained. Tissue damage occurs due to cytotoxic T cell responses, and inappropriate activation of macrophages (e.g. Insulin-dependent diabetes mellitus, Multiple Sclerosis, or Autoimmune hemolytic anemia), while the production of low-affinity antibodies against these structures results in an excessive formation of immune-complexes that contributes to the development of chronic inflammatory conditions (e.g. Systemic lupus erythematosus). In special cases of autoimmune responses, antibodies are produced against a receptor, and can block or trigger receptor mediated cell activation (e.g. Graves' disease, Myasthenia gravis).

It is also known that under inflammatory conditions an increase in the ratio of activating vs. inhibitory FcγR expression is induced within the effected tissue. Due to their function to down-regulate the immune response, a role of Fcγ-receptors in systemic autoimmunity was suggested. The first evidence for their importance in disease onset became obvious as Kleinau et al. reported that FcR γ-chain deficient DBA/1 mice were resistant to collagen induced arthritis (CIA), one of the most widely used animal models for rheumatoid arthritis; while the FcγRIIb knock out counterparts developed a more severe disease than wild type animals [63,64]. Bolland et al. and McGaha et al. showed that both in bone marrow chimeras and in a retroviral overexpression systems the correction of FcγRIIb levels mainly on B cells lead to the suppression of disease development in autoimmune-prone mice and FcγRIIb deficient animals. Surprisingly, the TD response was suppressed, but not the TI; an effect seen on the late IgG response, but not on the early IgM production (23).

The background of the manifestation of autoimmune disorders still remains unknown, however, there is a strong association between infection and the onset of disease. Genetic background and environmental issues also contribute to the development of autoimmunity.
Single nucleotide polymorphisms in the human RA and SLE were described in the trans-membrane part or in the promoter region of FcγRIIb and in the extracellular domain of FcγRIIa and FcγRIIIa, depending on the genetic background of the examined population (24) (25) (26). The dysfunction of the receptor might lead to the prolonged circulation of the immune complexes in the blood and impaired clearance of apoptotic fragments, which results in a maintained inflammatory response (27) (28). Also, reduced expression of FcγRIIb on

memory and plasmablast cells of SLE patients may result in decreased receptor mediated suppression of BCR-induced Ca^{2+}-influx in memory B cells (27).

According to a new aspect, B cell selection in the germinal center response may depend on the relative strength of opposing signals from the BCR and the FcγRIIb. FcγRIIb engagement via binding of immune complexes on germinal centre B cells with low affinity for antigens, or B cells with potential autoreactivity predominates over BCR engagement of antigens in the same immune complexes, thus the cells proceed to apoptosis. High affinity antigen-containing complexes on the contrary, inhibit proliferation and apoptosis, enabling the progression to memory B cells (29)

NK cells express predominantly FcγRIIc and FcγRIIIa; the former triggers the lysis of antibody-covered target cells and cytokine production, while the latter is mainly responsible for antibody dependent cellular cytotoxicity (ADCC). In RA-patients, an allelic polymorphism has been reported, which allows the expression of the inhibitory FcγRIIb instead of FcγRIIc on NK cells with a higher frequency, thus being able to modulate the stimulatory functions of other NK receptors and inhibit NK cell activation (30).

Many therapeutic approaches involve application of antibodies, including truncated or genetically modified immunoglobulin molecules like single chain antibody fragments.

1.5 Fcγ–receptor mediated signalling pathways

Similarly to the BCR- and TCR-mediated signalling processes, upon cross-linking of the ligand-binding extracellular domains of activating FcγRs, the ITAM gets phosphorylated by members of the src kinase family. In NK cells, FcγRIIIa aggregation leads to the activation of leukocyte-specific protein tyrosine kinase (lck), while the FcγRIIa- and FcγRIIIa-mediated activation of monocytes and mast cells is followed by lyn and hck phosphorylation .

Src kinases then phosphorylate the tyrosine residues in the ITAM sequences (pYXX[L/V]X6–9pYXX[L/V]), which results in the recruitment and activation of SH2 domain containing proteins (6), e.g. members of the syk kinase family: ZAP70 in NK cells and syk kinase in mast cells and monocytic cells. Activated syk kinases mediate the phosphorylation of a number of other proteins, initiating thereby a set of further signalling events.

Due to the activity of the phosphatidyl-inositol-3 kinase (PI-3K), PIP_3 gets enriched in the cell membrane, and facilitates the recruitment of PH domain containing adaptor molecules, e.g. SLP-76 or BLNK (B-cell linker protein). As a next step of downstream events, PLCγ and Tec kinases (Bruton's tyrosine kinase, Btk and IL-2 inducible T-cell kinase, Itk) are activated, which then leads to the generation of the second messengers inositol-3 phosphate (IP3) and diacyl-glycerol (DAG), and finally to Ca^{2+}-mobilisation (79). The phosphorylation of Tec kinases can also result in the activation of the mitogen-activated protein kinase (MAPK) cascade (31).

Phenotypic changes as a result of small G-protein involvement include cytoskeleton rearrangement, degranulation, ADCC and phagocytosis, followed by transcription of cytokine genes and inflammatory cytokine release.

The inhibitory FcγRIIb receptor displays its effect in different ways: (a) upon cross-linking with an activating receptor, e.g. BCR or FcεRI, the ITIM gets phosphorylated by Lyn kinase, and recruits the SH2-domain containing-5-inositol phosphatase 1 (SHIP-1), the SH2-domain containing tyrosine phosphatase 1, and the adaptor protein Grb2 (Growth factor receptor associated binder 2) proximal to the membrane. Lyn then phosphorylates SHIP-1 on the NPXY tyrosine, creating thereby a docking site for the PTB domain of the adaptor Dok-1, and once this complex is formed, another phosphorylation step on the C-terminal YXLP sequence of SHIP-1 occurs. This motif is a binding site of the SH2 domain of SHIP-1 therefore a competition is initiated between the FcγRIIb and Dok-1 for SHIP-1 binding. A mobile heterodimer is formed, which is able to hydrolyse PIP_3, and causes the release of

several PH domain anchored proteins, like Tec kinases and Akt from the cell membrane, which among other processes leads to a reduced Ca^{2+} signal. SHP-1 mediates the dephosphorylation of activated tyrosine kinases, e.g. Syk or Btk. As the SHIP-1/Dok-1 complex might possess high mobility, Cady et al. hypothesise that it is free to engage in a range of different interactions and hydrolyse its substrates (32) (80). SHP-1 on the other hand exerts only a local inhibition of signalling. (b) Phosphorylated Dok-1 also binds and activates RasGAP, inhibiting thereby the activation of the small G-protein Ras by increasing its intrinsically low catalytic activity causing GTP hydrolysis and rapid inactivation. Besides Ras, other members of the family of small G-proteins are regulated through the Grb2-pITIM interaction e.g. Rho and Rac. As a result of impaired MAPK phosphorylation, the cell cycle and the proliferation are affected. (c) The third supposed inhibitory mechanism characteristic only for B cells is independent of the ITIM sequence: upon homo-aggregation of the receptor a pro-apoptotic signal is generated via the c-Abl-family kinases. All these processes contribute to the regulation of cell activation, and the maintenance of a balance in the immune response (7, 8).

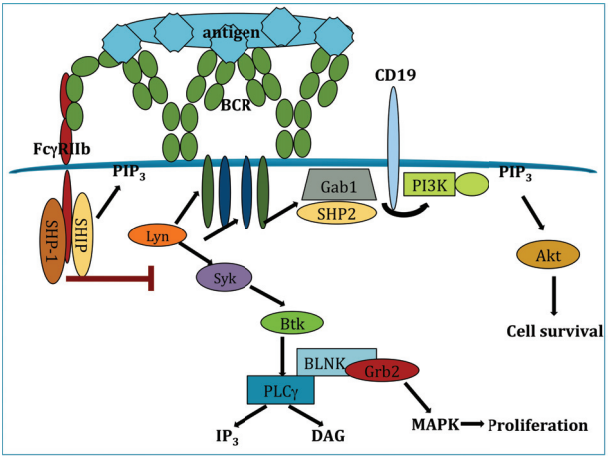


Figure 2.: Inhibition of antigen receptor mediated signalling events in B cells following cross-linking of BCR and FcγRIIb.

1.6 Single chain Fragment variable – a useful tool for investigating immune complex function

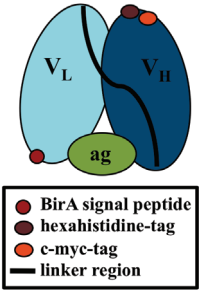
Single chain Fragment variables (scFv) are genetically modified recombinant antibody fragments consisting of the variable regions of the immunoglobulin (Ig) heavy and light chains (33). The two immunoglobulin-domains are usually connected by a serine-glycine containing linker region, which enables the correct conformation of the antigen-binding surface to be established. The specificity and affinity of the scFvs towards the antigen is usually equal to the original antibody, however, the avidity is reduced. As the construct lacks the Fc-fragment that mediates binding to Fc-receptor expressing cells, it is only the antigen-specificity of the single chain fragment that directs the protein to the targeted cells (34). Therefore, the application of scFvs could be of advantage in many processes including e.g. a more successful cellular targeting of drugs to tumour cells, ligating cell surface receptors in order to modify co-stimulatory signals or neutralisation of soluble mediators of inflammation (35). Due to their small size, they can easily penetrate into tissues, although the specific uptake is usually lower, as it was shown with radioactively labelled compounds. The half-life of an scFv construct is usually short, and it is filtered out from the circulation within hours. This can be modified e.g. with the co-expression of the CL/CH (constant domain of the light or the heavy chain) region, with generation of oligomeric complexes, or even with synthetic modifications like conjugation of polyethylene glycol chains (PEGylation) to the fragment (36). To the contrary, if reduction of the half-life of an antibody drug is necessary, e.g. for radioactive targeting, scFv molecules can be ideally used. Single chain Fragment variables, like other antibody-derived antigen-recognising molecules can be produced in mg amounts in bacterial protein expression systems, e.g. in *E. coli* cells, even in inclusion bodies or as intact periplasmatic proteins. Purification process of inclusion bodies includes several refolding steps, but the end product still contains partially refolded or unfolded molecules, and therefore offers a surface for aggregation and increases the instability of the protein. The preparations are usually contaminated with high levels of lipopolysaccaride (LPS) from bacterial cell wall origin.

Immunogenicity is a major issue regarding to all monoclonal antibody therapies as the foreign immunoglobulin is applied in high amounts, and a widespread and repeated application is therefore severely limited. Different strategies evolved to avoid the immune response that follows the treatment, offering a wide variety of choices from chimeric antibodies to transgenic mice. scFv constructs provide several advantages in this aspect as

they are less immunogenic due to lack of glycosylation on the variable region, and they can easily be modified by humanising the variable region frame sequences (37, 38).

Recent applications of human monoclonal antibody derived single chain fragments include e.g. trials for neutralisation of the influenza virus H5N1 (39), inhibition of T cell activation and autoimmune diabetes using a B cell surface-linked CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) agonist (40), or induction of programmed cell death in Her2-expressing tumour cells via targeted delivery of apoptosis inducing factor (41). Modeling of immune complexes, as described in the current work, is a specific way of the usage of cellular targeting.

(a)



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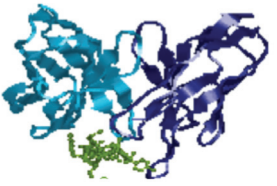

Figure 3.: (a) Schematic representation of an scFv, (b) structural model of the 7G6 scFv molecule–established with the RasMol program (figure drawn by Dr. Jozsef Prechl MD, PhD). Dark blue represents the V_H region, light blue is the V_L region, and green colour stands for an antigen caught trapped in the antigen-binding groove formed by the two Ig-domains.

2. Aims of this study were:

- to produce and *in vitro* characterise an scFv molecule from the mouse CD16/32 specific clone 2.4G2,
- to examine the target cells of the recombinant protein *in vivo,* after intravenous injection,
- to establish a model immune complex from the biotinylated 2.4G2 scFv and FITC, as a hapten,
- to investigate the role of these immune complexes on T-independent and T-dependent humoral immune response *in vivo,*
- to study whether it is possible to target B cells via the FcγRII by using these immune complexes and inhibit their activation (potential therapeutic importance in systemic autoimmune disorders).

3. Materials and Methods

3.1 Cell culture

All mouse cells were cultured in RPMI 1640 supplemented with 5% foetal calf serum in the presence of β-mercapto-ethanol (RPMI 1640: 15.36 g/l, $NaHCO_3$: 2 g/l, Na-pyruvate: 110 mg/l, glutamine: 292 mg/l, β-mercaptoetanol: 0.1 M, streptomycin: 50 mg/l, penicillin: 100000 NE/l, foetal calf serum: 5v/v%) at 37ºC under a 5% CO_2 containing atmosphere.

3.2 Mice

C57BL/6 mice at the age of 6-16 weeks were used for all experiments. Previously characterised CD16KO animals were a kind gift from Dr. Attila Mocsai. Animals were fed a standard diet and tap water ad libitum. Ethical approval was obtained from the local research ethic committee.

3.3 PCR synthesis and cloning of the scFv

The rat anti-mouse CD16/CD32 specific clone 2.4G2 was kindly provided by the Department of Immunology, University Hospital Utrecht, The Netherlands.
Cells were cultured under standard conditions in supplemented RPMI 1640 and the supernatants were tested for antibody content and specificity. Total RNA was isolated from the cells using the Qiagen RNeasy kit and reverse transcription was performed with the RevertAid™ M-MuLV Reverse Transcriptase enzyme (Fermentas) using oligo dT primer, both according to the manufacturer's instructions. Immunoglobulin heavy and light chain variable sequences were amplified using PCR with the following primers, which already contained the Nco I-Not I restriction sites:

VH3NotI: 5' - TGC GGC CGC GGA GAC GGT GAC CGG- 3'

Vk3Cut: 5' - TTT GAT TTC CAC CTT GGT CCC – 3'

VkBackNco: 5' - TCC ATG GAC ATT GAG CTC ACC CAG TCT CC – 3'

CHγ: 5' - GGA TAG ACA GAT GGG GCT GTT G - 3'

The thermal cycling profile of the reaction:

94°C 3’ denaturation
55 °C 2’ annealing
---
72 °C 30”
94 °C 30” 30 cycles
55°C 30”
---
72 °C 10’ final extension.

PCR fragments were run in a 1.6% low-melting point agarose gel in TAE buffer (Tris-acetate-EDTA buffer: 4.84 g/l TRIS.BASE, 2.723 g/l Na-acetate x $3H_2O$, 0.372 g/l Na_2EDTA x $2H_2O$) in the presence of 5 μg/ml ethidium-bromide, then isolated and purified with the MinElute Gel Extraction Kit (Qiagene), according to the manufacturer’s instructions. In order to achieve overlapping regions that encode the linker region of the scFv and secure it’s flexibility, the 3’ strand of the light chain and the 5’ strand of the heavy chain sequences had to be modified with the following primers:

Vκ3Link: 5’ AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC TTT GAT TTC CAC CTT KGT SC 3’
LinkCHγ: 5’ GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG CAG GTG CAG CTG SWG GAG TC 3’
The fusion of the two fragments was performed in an overlap-extension step, under the following conditions:
The thermal cycling profile of the reaction:

94°C 3’ denaturation
57 °C 2’ annealing
---
72 °C 60”
94 °C 30” 30 cycles
55°C 30”
---
72 °C 10’ final extension

The construct was first prepared for cloning as described above, and ligated with T4 DNA ligase into an EcoRV linearised pBluescript KS cloning vector. The reaction contained 1μl 10x buffer, the vector and the purified insert in a 1:5 molar ratio (0.1-0.5 μg each), 0.5 μl EcoRV (Fermentas) and 1 μl T4 DNA ligase (Promega), added up with water to 10 μl final volume. 100 μl of E. coli DH5α competent cells was thawed on ice and incubated with 5 μl ligate for 15 minutes, then placed to 42°C for 45 seconds. Sequentially, 900 μl LB medium (10 g trypthon, 5 g yeast extract, 10 g NaCl, 100 μg/ml ampicillin) was added and the cells were cultured at 37°C for 30 minutes. Transformed bacteria were then spread to a 40 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 0.1 mM IPTG containing 1.5% agar plates. White colonies were picked up and screened by restriction digests, then the purified plasmid samples were cut at the NcoI-NotI sites, isolated, and cloned into a linearised pET11d vector encoding a hexahistidine-, and a myc-tag at the C-terminal region. The intactness of the construct was verified by nucleotide sequencing, the analysis of the chromatograms was performed with DNASTAR, and further analysis of the heavy and light chain variable region sequences were accomplished by using NCBI-BLAST and the Kabat sequence database.

3.4 Purification, refolding and enzymatic biotinylation of the recombinant scFv protein

At the purification and refolding steps the method described by Kurucz et al was applied (42). After sequencing, the plasmid construct was transformed into BL21 competent cells, and a starter culture was grown overnight at 37°C in 100 μg/ml ampicillin containing LB, with constant shaking. The next day, a fresh culture was inoculated at 1:100 dilution and grown at 37°C until it reached OD_{600nm}=0.6-0.8. Protein expression was then induced by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), a constitutive inducer of the lac operon system and cells were cultured for 3 more hours. Bacteria were pelleted, resuspended in STE (100 mM Tris-HCl,-2 mM EDTA) at 10ml/g wet pellet, in the presence of 10 mg/ml lysozime, and incubated on ice for 30 minutes. After three sonication and washing steps, the pellet was solubilised in 50mM Tris-HCl (pH=8) solution containing 2% sodium lauroylsarcosin (SLS), then supplied with 10 μM $CuSO_4$, and stirred overnight with a loose cap to achive the correct formation of intramolecular disulphide bounds. The next day, urea was added to the protein solution at 6 M concentration, and the detergent (SLS) was removed with anion exchange DEAE-Sephadex beads. The solution was passed through an equilibrated Ni-NTA-agarose column, and the refolding was promoted by washing with buffers containing

decreasing urea (6-1 M), and increasing NaCl (0-300 mM) concentrations. Following a washing step with a wash buffer (10 mM imidazole, 1% glycerol, 300 mM NaCl and 50 mM Tris-HCl pH=8.5), the scFv was eluted from the column (elution buffer: 250 mM imidazole 300 mM NaCl and 50 mM Tris-HCl pH=8.5).

Protein constructs containing a fusion peptide-tag recognised by the *E.coli* biotin protein ligase (43) (44) (BirA, Avidity) were biotinylated enzymatically: the refolded 2.4G2 scFv (optimal concentration 1mg/ml) was dialysed into 10mM Tris-HCl (pH=8.0), then mixed with Biomix A (10x concentration, 0.5 M bicine buffer, pH=8.3) and Biomix B (10x concentration, 100 mM ATP, 100 mM MgOAc, 500 μM d-biotin), finally, 1μl of BirA was added, and the reaction was incubated overnight at RT. The next day the excessive biotin was removed by gel filtration chromatography, and the efficiency of biotinylation was analysed with SDS-PAGE gel electrophoresis and flow cytometry.

3.5 SDS-PAGE and dot blots

Protein fractions following Ni^{+}-NTA column based purification or following removal of the excessive biotin after the enzymatic reaction were checked for purity and integrity by running the samples on a 15% SDS-polyacryl-amide gel, followed by horizontal transfer of the proteins at 200 mA constant current for 90 minutes at RT to nitrocellulose membrane or by staining with Coomassie Brilliant Blue solution for an hour at RT, where the non-specific stain was removed with differentiation buffer in several washing steps thereafter.

0.5μg of the purified protein 2.4G2 scFv, 7G6 scFv or bovine serum albumin was bound to nitrocellulose membrane, then the dot blot was rinsed with TWB-0.05% Tween and blocked in 5% milk powder containing wash buffer for an hour at RT. The membrane was then incubated for an hour at RT with the c-myc specific primary antibody clone 9E10 (M4439 Sigma Aldrich) diluted in 5% milk powder at 1 μg/ml concentration. After a 3x5 minutes washing step, the secondary antibody (rat anti-mouse IgG1-HRPO, Southern Biotech 1070-05) was diluted in 1:3000 in blocking buffer and applied under the same conditions. The Western blot was developed with the Enhanced Chemiluminescent (ECL) Western Blotting Substrate (Pierce) on Medical X-Ray Film General Purpose Blue (MXB, Kodak).

Solutions and buffers for SDS-PAGE gel electrophoresis and dot blot analysis:

-10x Tris-glycine electrophoresis buffer

Tris-HCl	0,25 M
Glycine	1,92 M

-Running buffer

10x-es Tris-glycine-buffer	100 ml
10% SDS	10 ml
Milli Q water	890 ml

-Resolving gel (15%)

30% acryl-amide/bis-acryl-amide	2.31 ml
Milli Q water	1.37 ml
1.5 M Tris-HCl (pH 8.8)	1.25 ml
10% SDS	50 µl
10% APS (ammonium-persulphate)	25 µl
TEMED (tetramethyl-ethylendiamine)	2.5 µl

-Stacking gel (4%)

30% acryl-amide/bis-acryl-amide	0.67 ml
Milli Q water	3.05 ml
0.5 M Tris-HCl (pH 6.8)	1.25 ml
10% SDS	50 µl
10% APS	25 µl
TEMED	5 µl

-2x reducing sample buffer

0.5 M Tris/HCl (pH 6.8)	1 ml

10% SDS (Na-dodecyl-sulphate)	1.6 ml
2-mercapto-ethanol	400 µl
glycerine	800 µl
bromo-phenol blue (10w/v% stock)	200 µl

-Wash buffer (TWB-0.05% Tween-20)

NaCl	80 g/l
Tris (tris(hydroxi-methyl)-amino-methane)	24.22 g/l
Tween-20	500µl/l

3.6 Flow cytometric analysis of the binding characteristics of 2.4G2 scFv

Flow cytometry was performed on the mouse B cell line, A20 and on splenocytes isolated from C57Bl/6 mice, respectively. Spleens were removed and treated first with 2 mg/ml collagenase D (11088858001, Roche) in collagenase D digestion buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1mM $MgCl_2$, 1.8 mM $CaCl_2$, pH=7.4) for 30 minutes at 37˚C, then smashed and cell suspensions were haemolysed and filtered with 40µm cell culture strainer (BD 352340).

$5*10^5$ A20 cells or splenocytes were washed and resuspended in 100 µl flow buffer (PBS supplemented with 1% BSA and 0.1% NaN_3), then labelled with 10 µg/ml of biotinylated monomeric 2.4G2 scFvs followed by detection with 0.1 µg/ml FITC-conjugated avidin (Sigma), or with preformed biotinylated 2.4G2 scFv-avidin-FITC complexes (molar ratio 4:1) for 15 minutes on ice. Alternatively, the binding of 2.4G2 scFv was detected by 0.1µg/ml streptavidin-Alexa488 (Invitrogene). In some experiments cells were simultaneously stained with rat anti-mouse CD45R (B220)-PerCP/Cy5.5, rat anti - mouse CD11b-Alexa 647 (eBioscience) or anti-mouse CD11c (Serotec), respectively, followed by washing steps with 1% FCS and 0.01% sodium-azide containing PBS after each staining steps. Samples were analysed by flow cytometry (FacsCalibur, Beckton Dickinson), and data were evaluated with the winMDI 2.8 software.

3.7 Immunisation of mice

Mice were immunised intraperitoneally (i.p.) with 100 μg FITC-dextran (average mol wt 70000 Da) diluted in 200 μl PBS (T-independent antigen) or 50 μg FITC-conjugated keyhole limpet hemocyanine (KLH-FITC) in 100 μl PBS mixed with 50 μl complete Freund's adjuvant/animal (T-dependent antigen) on day 0. Biotinylated 2.4G2 scFv-avidin-FITC (b-scFv-avidin-FITC) complexes were injected intravenously on day 3 after primary immunisation. The complexes were preformed by mixing 60 μg biotinylated 2.4G2 scFv (b-scFv) with 30 μg avidin-FITC, and incubated at room temperature for 15 minutes. In the case of T cell dependent B cell response a second injection of the complexes was performed on day 6. Mice were sacrificed 3 days after the last injection and the number of FITC-specific cells in the spleen was determined by ELISPOT assay. Statistical analysis of the data was performed by GraphPad Prism 4.

3.8 ELISPOT assay measuring FITC-specific antibody producing cells

ELISPOT MultiScreen-HA Filter Plates (Millipore MAHAS4510) were coated with 100 μl 5 μg/ml FITC-BSA dissolved in PBS overnight at 4ºC, and then the wells were washed three times with 200 μl PBS under sterile conditions. Wells were blocked with 200 μl 10% FCS containing RPMI 1640 for an hour at 37ºC. Spleens of treated animals were removed, smashed and treated with 2ml haemolysis buffer for 1 minute in order to remove red blood cells from the cell suspension. After the removal of the blocking solution, 10^5 or $5*10^5$ splenocytes/well were seeded to the plates and incubated for 16 hours at 37ºC. The plates were then washed three times with PBS, another three times with PBS- 0.05% Tween-20 and membrane bound IgM and IgG containing spots were detected with HRP-conjugated rabbit anti-mouse IgM and IgG. For the assay-development 10 mg aminoethyl-carbazole (AEC, Sigma Aldrich) was dissolved in 1ml of DMF, and filtered after 5 minutes incubation at RT, then the filtrate was diluted in 30ml 0.1M sodium-acetate buffer (pH 5.5) and supplemented with 15 μl of 30% H_2O_2. Precipitate formation indicated the site of antigen producing B cells on the membrane. Number of spots was evaluated by the C.T.L. ImmunoSpot Professional Software Version 4.0 for ELISPOT analysis.

3.9 Immunofluorescencent detection of the *in vivo* localisation of b-2.4G2 scFv-avidin-FITC complexes

Spleens of b-2.4G2 scFv-avidin-FITC complex treated or FITC-dextran immunised and complex-treated animals were taken and mounted in cryostat embedding medium (Killik, Bio-Optika, Milan, Italy), then stored at –80ºC until being processed. Frozen sections of 8 μm thickness were cut, collected and fixed in ice-cold acetone for 5 minutes, then blocked with 5% BSA in PBS for 30 minutes at RT in wet chamber. Sections were incubated at RT in wet chamber for 1h with antibodies specific for mouse c-myc tag (purified at the Department of Immunology, Eotvos University), marginal zone macrophage marker (MARCO) (45) or a vein endothelial marker recognising antibody IBL-9/2 (46) (both purified at the Department of Immunology, Medical University of Pecs), all diluted in blocking buffer at a final concentration of 1-5 μg/ml. After 3x 5 minutes washing with PBS, PE-labelled polyclonal rabbit anti-rat IgG was used for the detection of the primary antibodies, fluorescent images were captured using a ColorView CCD camera mounted onto an Olympus BX61 fluorescent microscope.

4. Results

4.1 Cloning of the 2.4G2 IgG light and heavy chain variable region genes, synthesis of the scFv construct

The monoclonal rat IgG2b clone 2.4G2 was shown to recognise a common polymorphic epitope on the mouse CD16/32 receptors, expressed on a wide range of leucocytes e.g. monocytes, macrophages, granulocytes, NK cells, DCs, mast cells, B-lymphocytes and $CD4^-/CD8^-/TCR^-$ thymocytes. The aim of this work was to establish a single chain Fragment variable construct by cloning the V_L and V_H sequences of the 2.4G2 antibody, and produce the recombinant protein in a bacterial expression system in order to further examine the effect of immune complexes on the humoral immune response.

2.4G2 hybridoma cells were cultured under standard conditions, then cells were collected, and the total RNA was isolated and used for reverse transcription, primed by oligod$T_{(18)}$. This cDNA served as a template in the polymerase chain reaction, where we obtained a V_L fragment of 321 bp and a V_H fragment of 363 bp (Figure 4.a).

The whole construct was then produced by the fusion of the two fragments (Figure 4.b) and the insert was cloned into two differently modified pET11d expression vectors, one of them encoding two additional peptide-tags at the C-terminal part of the sequence: a hexa-histidine tag that facilitated effective purification and refolding, and a c-myc derived peptide of 9 amino acids that was used for the detection of the recombinant protein. This vector enabled us to produce monomeric scFv, while the other vector also containing a signal peptide for the biotin ligase BirA at the N-terminal part was applied for establishing the model complexes. Partial *in vivo* biotinylation of the unfolded protein expressing this tag already happens due to the presence of the BirA enzyme in E.coli used for the production of the recombinant construct, therefore, we had to work with both of these plasmids in some of the experiments.

The schematic maps of the final constructs used in this work are shown in Figure 4.c.

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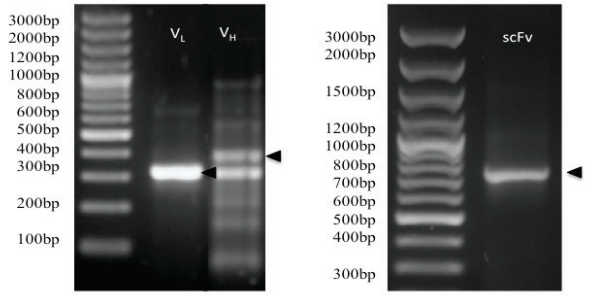
(a) (b)



(c)

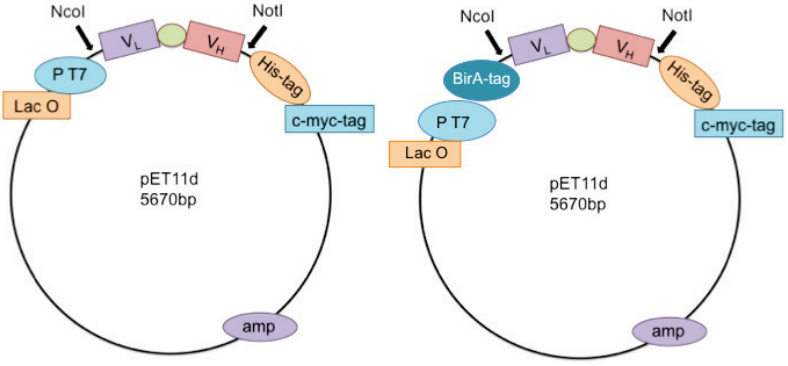


Figure 4.: After the amplification of the light and heavy chain variable region sequences as described in section "Materials and methods", samples were run on a 1.6% agarose containing TAE gel (a). Figure 4. (b) shows the PCR-product after the overlap extension step, where the fragment was run in a 1% agarose gel. (c) shows the schematic maps of the scFv construct in different pET11d expression vectors.

4.2 Nucleic and amino acid sequence analysis of the 2.4G2 scFv construct

Bacterial colonies were screened via restriction digests, and the clones showing the correct restriction patterns were sequenced. Nucleic acid sequences were analysed with Lasergene (DNASTAR, Madison, WI, USA) and with an on-line sequence analysis tool 'The Sequence Manipulation Suite' (www.ualberta.ca/~stothard/javascript, University of Alberta, Canada) for open reading frames (Figure 5.).

The translated amino acid sequences were further compared with the Kabat database of immunoglobulin genes. We could identify the essential amino acids in the framework sequences including the cysteine residues in positions 23 and 88 in the light chain, and 22 and 92 in the heavy chain, which are responsible for the intra-molecular disulphide bond formation within the immunoglobulin domain. We could also identify the complementarity determining regions (CDR1-3, green boxes), thus the cloned sequence corresponded to a fusion protein of the rat immunoglobulin light and heavy chain variable regions.

The blue box on Figure 5. marks the linker region that assures the structural flexibility within the two domains. Finally, the sequences were submitted to the NCBI Nucleotide Database as a direct submission (GenBank: FJ888350.1 for the light chain, and GenBank: FJ888349.1 for the heavy chain).

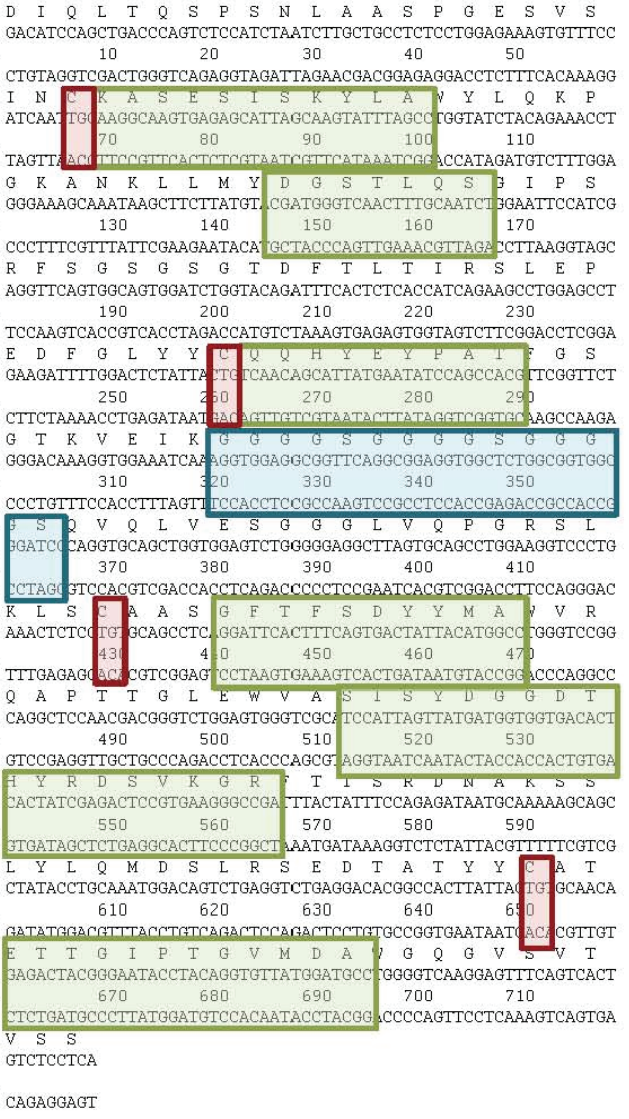


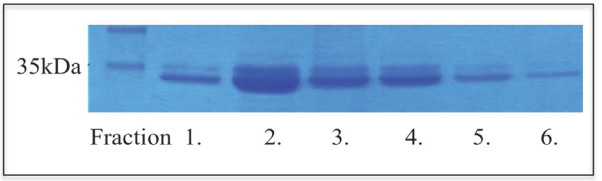
Figure 5.: Nucleic-, and amino acid sequence of the 2.4G2 scFv construct (red boxes indicate the cysteine residues essential for disulphide bridge formation, green boxes show the complementary determining regions and the blue box highlights the linker region between the V_L and V_H regions). The figure was designed by using 'The Sequence Manipulation Suite' (www.ualberta.ca/~stothard/javascript).

4.3 Production and refolding of the recombinant protein

The recombinant scFv was purified from bacterial inclusion bodies as described in 'Materials and methods', and the protein fractions were analysed via SDS-PAGE on a 15% acryl-amide-bis-acryl-amide gel. The Coomassie Brilliant Blue staining revealed a 35kDa protein, being excessively represented in the main fractions 2, 3 and 4 (Figure 5.a), indicating that the recombinant protein was successfully purified.

Samples were also tested for c-myc tag expression: 1µg of BSA, the 2.4G2 scFv or the 7g6 scFv was bound to nitrocellulose membrane, and the dot blot was developed with a c-myc specific primary antibody under standard conditions (Figure 5.b). The majority of our experiments required the usage of biotinylated scFv therefore we had to modify the purified protein in an additional enzymatic biotinylation step. The effectiveness of this process was checked by flow cytometry for every single lot individually.

(a)



(b)

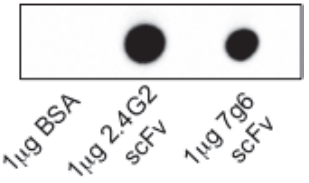


Figure 6.: (a) Fractions of 2.4G2 scFv after purification and refolding on a NiNTA column (SDS-PAGE with Coomassie Brilliant Blue staining).
(b) Dot blot analysis of the purified protein, scFv constructs detected with the c-myc specific clone 9E10, and a HRP-conjugated goat anti-mouse IgG-specific secondary antibody (chemiluminescent signal on X-ray film).

4.4 Biotinylated 2.4G2 scFv (b-2.4G2 scFv) - avidin FITC complexes specifically bind to FcγRII/III receptors expressed on A20 mouse B cells or splenocytes *in vitro*.

Although the epitope recognised by the whole antibody is usually identical to the one recognised by the scFv derived from the same clone, first we had to check the functional integrity of b-2.4G2 scFv by characterising its binding abilities to different cells.
A20 mouse B cells were labelled either with the botinylated scFv followed by detection with avidin-FITC in a separate step (Figure 7.a, green) or –as we wanted to examine the effect of complexes on the immune response-, we also used the preformed complex of the biotinylated scFv and avidin-FITC (Figure 7.a, blue). Both procedures verified a significant binding of the b-2.4G2 scFv to mouse B cells.

In another set of experiments we checked whether the scFv construct is able to compete with the whole antibody (Figure 7.b., green) for FcγRII binding on A20 cells. The addition of excessive unlabelled scFv blocked the binding of the whole antibody to the cell surface (Figure 7.b, blue), demonstrating that the construct was functionally intact (baseline signal shown in red).

In vitro binding to splenocytes was assessed by simultaneous labelling of spleen cell suspensions for the B cell marker CD19 and for CD16/32 with the biotinylated 2.4G2 scFv, detected by avidin-FITC. Similarly to the whole antibody, the scFv also stained the majority of CD19 positive cells (Figure 7.c and d, binding of the whole antibody not shown). A proportion (4.08%) of $CD19^-$ cells corresponding to FcγRII/III-expressing myeloid cells represented in the spleen was also labelled by the scFv construct.

Taken together these data suggested that the established b-2.4G2 scFv is specifically recognising the mouse CD16/CD32 *in vitro*, therefore it resulted to be suitable for the further *in vivo* applications.

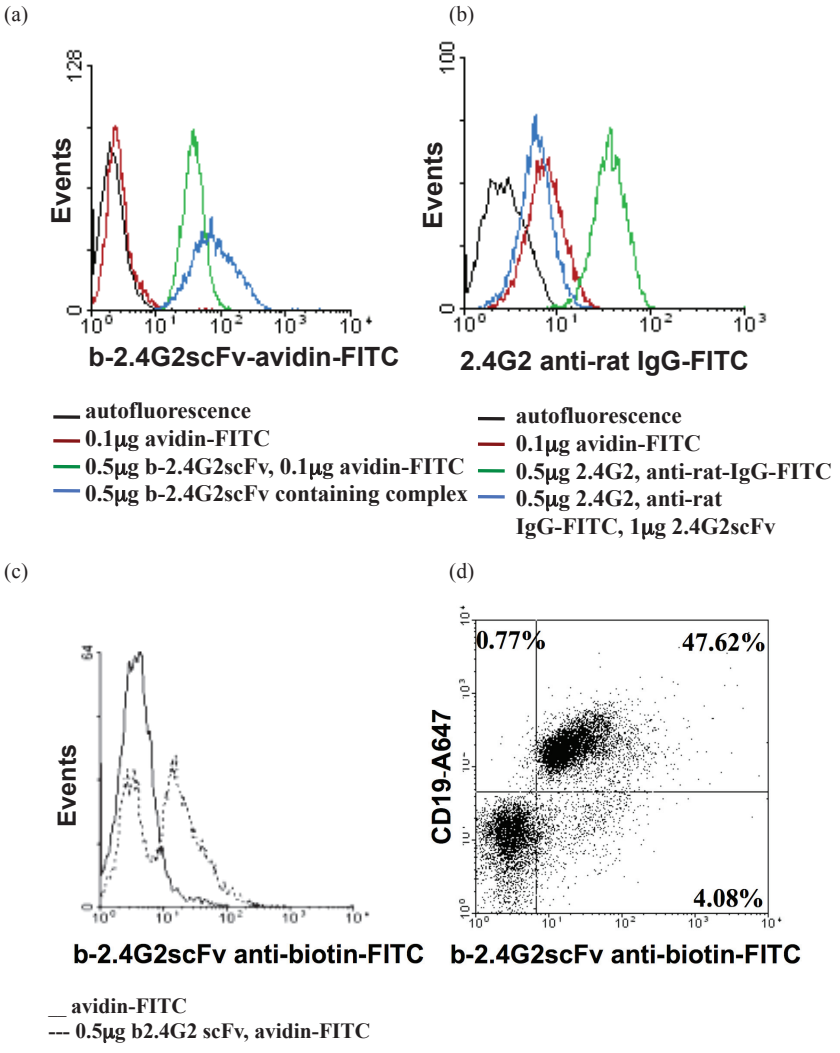


Figure 7.: (a) Binding of the biotinylated 2.4G2 scFv to A20 cells, (b) competition experiments between the 2.4G2 scFv construct and the whole antibody, (c and d) binding of the construct to splenocytes, where cells were co-stained for the B cell marker CD19 within the same experiment.

4.5 b-2.4G2 scFv-avidin FITC complexes bind to a subset of FcγRII/III receptors expressing splenocytes *in vivo*.

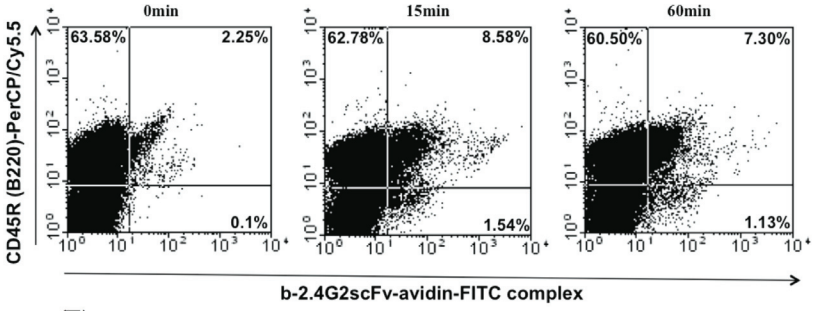
As our aim was to study the regulatory role of well defined immune complexes on the antibody production, we prepared preformed b-2.4G2 scFv-avidin-FITC complexes by mixing the components at 4:1 molecular ratio and tested the binding to spleen cells of naïve C57BL/6 mice *in vivo*, 15 min and 60 min after intravenous injection. Preliminary data indicated that FITC might be internalised, which results in a decreased fluorescent intensity, possibly due to low lysosomal pH-values. Therefore, we used the more stable Alexa Fluo488 dye for this single experiment.

Following collagenase D treatment of the isolated spleens, we co-stained the samples for different subpopulations. Partial binding of the preformed complexes was detected on B220+ cells (Figure 8.a), on CD11b+ cells (Figure 8.b), and on CD11c+ cells (Figure 8.c), respectively. Although we have tested many different conditions including increasing the amount of the injected protein, allowing the complex a longer time to circulate, or preparing cell suspensions without collagenase D digest, we could not detect a higher rate of complex binding to B cells under any of these conditions.

As our tests run on blood samples of the treated mice showed no B-cell CD16/32 staining in the selected time-points (data not shown), it seemed to be unlikely that the complex would not reach the spleen because of being trapped by blood cells. One possible explanation for the differences between the *in vitro* and *in vivo* data is that the complexes might be filtered out from the circulation by the marginal zone within minutes following the injection, thus follicular B cells never get into contact with the intact complex.

To confirm the flow cytometric data and gain some further information about the localisation of the complex, we went on with the experiments and performed immunofluorescent staining on frozen spleen sections of b-2.4G2 scFv-avidin-FITC complex injected animals.

(a)



(b) (c)

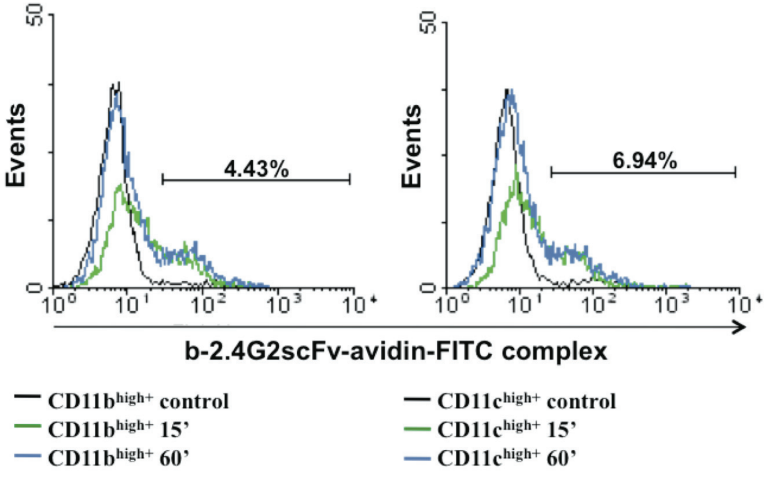


Figure 8.: *In vivo* binding of the b-2.4G2 scFv-avidin-FITC complex to (a) $B220^{+}$, (b) $CD11b^{high+}$ and (c) $CD11c^{high+}$ splenocytes. Data provided by Zs. Szekeres.

4.6 Localisation of the b-2.4G2 scFv-avidin FITC complexes in the spleen

In order to examine the localisation of the complexes in the spleen we injected naïve mice intravenously with the 2.4G2-FITC model immune complexes. Thereafter, 8 μm thick frozen spleen sections were prepared to see whether the endogenous dye is detectable. Our fluorescent images showed that 15 minutes after injection the complex was localised in the red pulp of the spleen, but was already partially internalised by marginal zone macrophages. 60 minutes after injection the signal was trans-located to marginal zone macrophages, and by the third hour it was completely diminished, probably due to antigen processing (Figure 9.a).
We also wanted to check that the fluorescent signal indicates the presence of the whole b-2.4G2 scFv-avidin-FITC complex rather then the accumulation of avidin-FITC after degradation of the complexes. Therefore, we stained sections for the c-myc-tag expressed on the scFv as well (Figure 9.b, spleen removed after 15 minutes of injection), and found that the signal.is strongly co-localised with the FITC-signal.

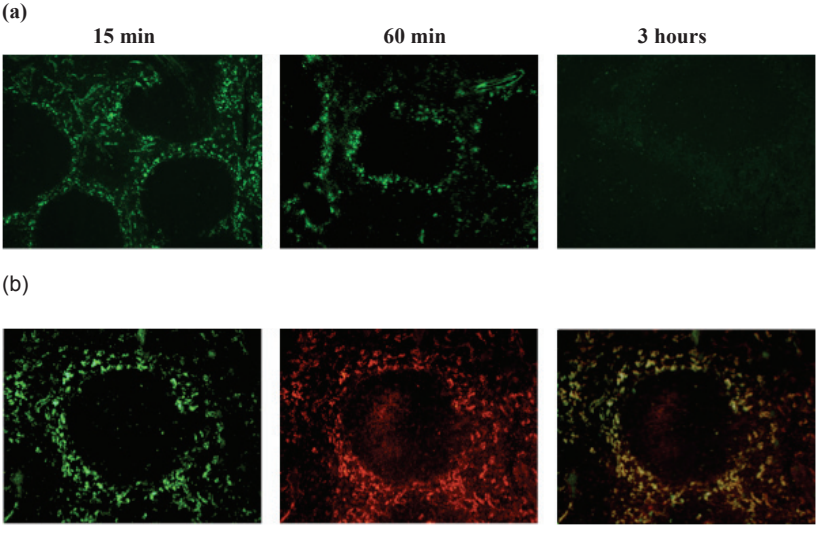
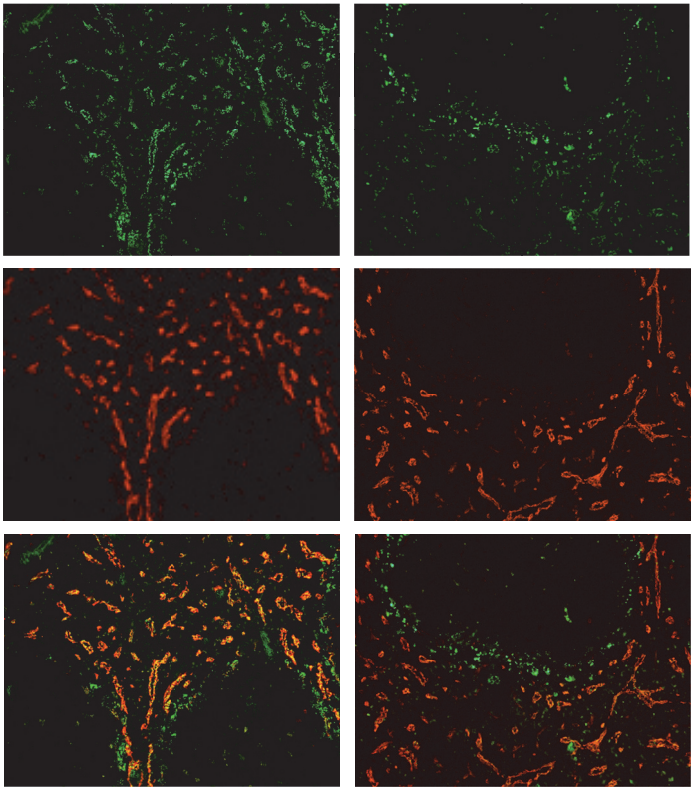


Figure 9.: (a) b.2.4G2 scFv-avidin-FITC complexes were injected intravenously to naïve C57BL/6 mice, and the spleens were removed 15 minutes, 60 minutes or 3 hours after injection. (b) Frozen sections were also stained for c-myc tag expression, 15 minutes following injection the complex (green) and the c-myc tag signal (red) co localised in the red pulp and the marginal zone (yellow).

To further identify the site of binding, we co-stained the sections for several markers including B-cell marker B220 and IgM, complement receptors CR1/2, red pulp endothelial marker IBL9/2 (Figure 10.a and b) and marginal zone macrophage marker MARCO (Figure 10.c and d). The b-2.4G2 scFv-avidin-FITC complex was first localised mainly on red pulp endothelial cells, then 60 minutes following treatment it was relocated to MARCO^{+} marginal zone macrophages.

(a) 15 min (b) 60 min



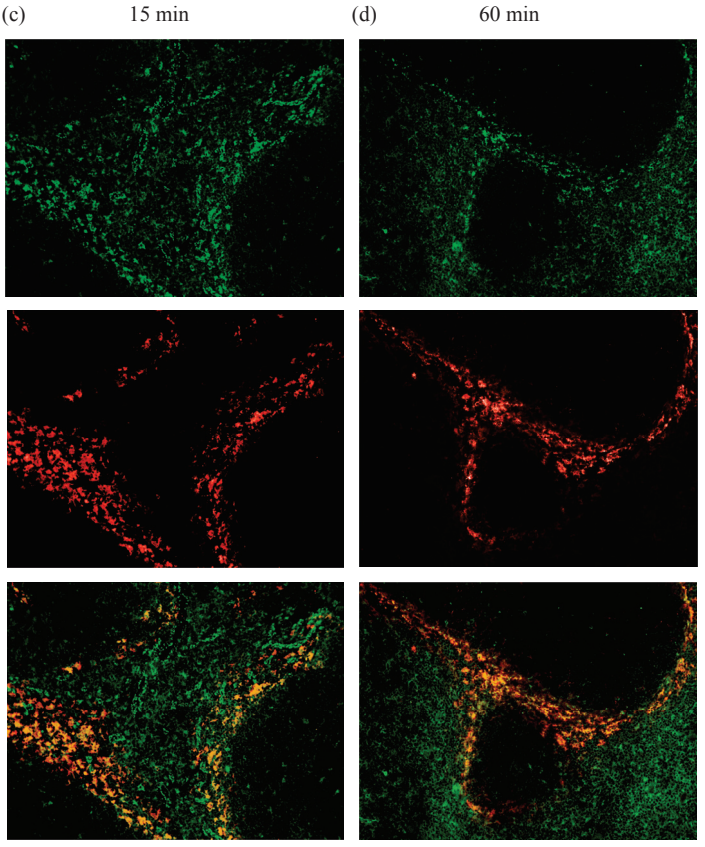


Figure 10.: (a) 15 minutes after injection the complex (green) is bound mainly by red pulp endothelial cells (red), and a partial binding can be observed in the marginal zone; while 60 minutes after intravenous treatment the 2.4G2 scFv complex is already mainly localised in the marginal zone (b), as shown by co-staining with the red pulp endothelial cell marker IBL-9/2. Co-staining with the marginal zone macrophage marker MARCO 15 minutes (c) or 60 minutes (d) after injection also supports this observation.

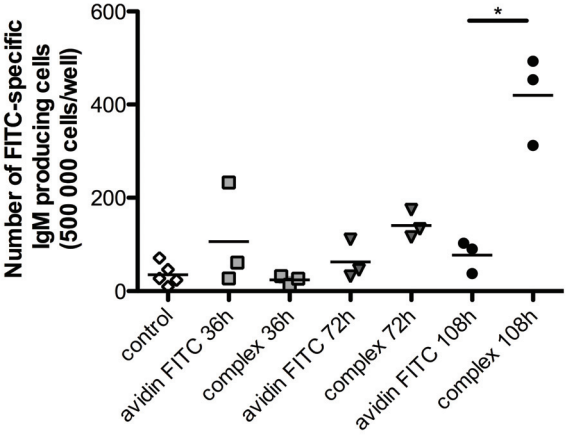
4.7 b-2.4G2 scFv - avidin FITC complexes time dependently increase the number of FITC-specific antibody forming cells in FITC-dextran immunised mice

We aimed to examine whether the complex formed by b-2.4G2 scFv-avidin FITC has any modulatory effect on the humoral immune response, namely, on the FITC-specific antibody production. To this object, wild type C57BL/6 animals were immunised with the T-independent 2 (TI-2) antigen, FITC-dextran to induce a fast and B-cell driven response, then the animals were treated with preformed complexes directing the hapten (FITC) to FcγRII and FcγRIII positive cells 36, 72 and 108 hours after primer immunisation. The number of antibody secreting cells was determined in an enzyme linked immunospot assay (ELISPOT) assay on day 6.

b-2.4G2 scFv-avidin FITC complexes time-dependently enhanced the antigen specific humoral response, resulting in a significantly higher proportion of FITC-specific IgM producing B cells at the 108 hours time point as compared to animals receiving only avidin-FITC injection (Figure 11.a). Number of antigen specific IgG producing cells showed a non-significant increase in this particular experiment (Fig.11.b).

Based on these data, in further experiments we decided to treat the animals with the complex on day 3 after primer immunisation.

(a)



(b)

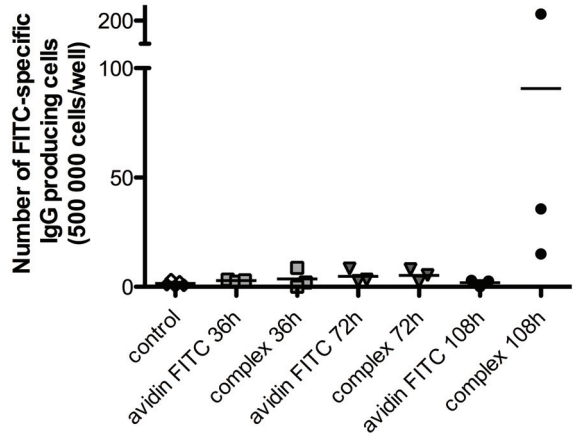


Figure 11.: Wild type C57BL/6 mice were intraperitoneally immunised with the TI-2 antigen FITC-dextran and treated intravenously at different time-points (36h, 72h and 108h after primer injection) with avidin-FITC or a pre-formed complex of biotinylated 2.4G2 scFv-avidin-FITC. Number of FITC-specific IgM (a) and IgG (b) producing B cells was measured by enzyme-linked immunospot assay (ELISPOT) on FITC-BSA coated nitrocellulose microplates 6 days after first antigen encounter.

4.8 The b-2.4G2 scFv-avidin-FITC complexes increase the FITC-specific humoral immune response by binding to FcγRII/III *in vivo*

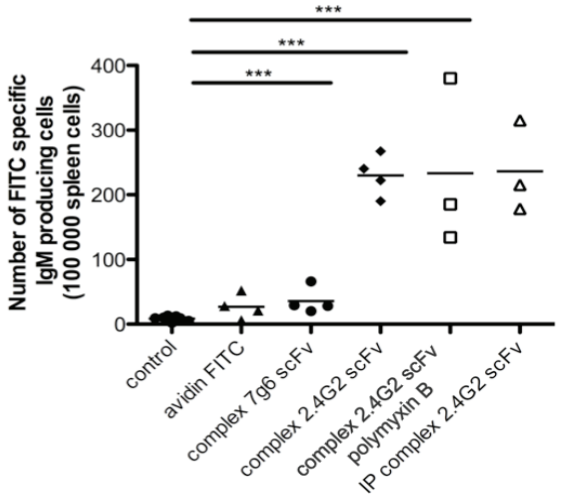
The recombinant proteins purified from bacterial expression systems contain high amounts of bacterial lipopolysaccharide (LPS), which is able to trigger the immune system if injected to an animal; independently from the features of the recombinant protein, in our case the specificity of the scFv. Therefore, in the next set of experiments we aimed to investigate the importance of LPS in the 2.4G2 scFv mediated modulation of the immune response and tried to prove that the observed effect is caused by the ligation of CD16/32 rather then by the single chain structure.

Our controls involved groups injected with avidin-FITC and additional LPS at a concentration of 1μg/ml (▲), polymyxin B treated recombinant scFv, which supposed to neutralise LPS (□), or usage of another biotinylated scFv-avidin FITC containing complex, obtained from the mouse CR1/2 specific clone 7g6 (●). The site of booster injection was also changed; a group of mice was challenged with the complexes intraperitoneally instead of intravenous (Δ) injection (Figure 12.).

The results showed that additional LPS did not modulate significantly the response, nor did neutralisation of LPS reduce the number of FITC-specific IgM/IgG producing cells. The complex formed by 7g6 scFv and avidin FITC did not have any significant influences, either. The intraperitoneal booster induced the same effect as the intravenous treatment, namely an increment in the antigen-specific antibody response. This indicates the involvement of soluble factors in the mechanism, e.g. cytokines besides the role of complex binding APCs.

These data show that FcγRII/III-specific targeting of immune cells has a positive effect on the immune response elicited by the TI-2 antigen.

(a)



(b)

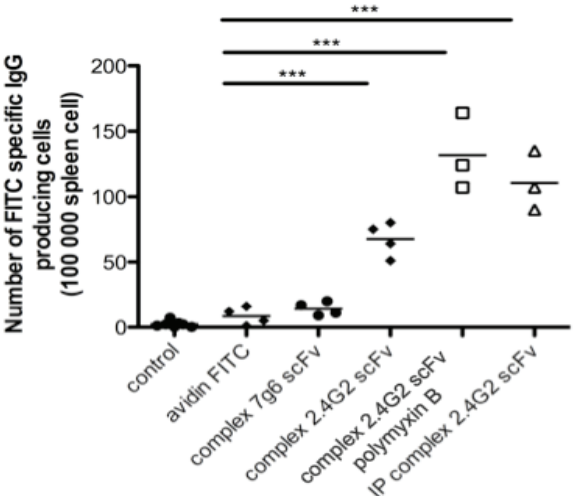


Figure 12.: Receptor specificity of the immune response enhancing effect of biotinylated 2.4G2 scFv-avidin-FITC complex was investigated via different approaches. (a) shows the number of FITC-specific IgM producing cells after treatment with avidin-FITC (supplemented with additional LPS at a concentration of 1 μg/ml, ▲), with the preformed complexes of biotinylated CR1/2 specific scFv 7g6-avidin FITC (●) or biotinylated 2.4G2 scFv-avidin FITC (with (□) / without (◆) the LPS neutralising reagent polymyxin B), injected i.v. or biotinylated 2.4G2 scFv injected i.p. 4 days post-immunisation with FITC-dextran. (b) shows the number of FITC-specific IgG producing cells in the same experiment.

4.9 The b-2.4G2 scFv-avidin-FITC complexes induce an increased FITC-specific humoral immune response both against T-cell independent 2 (TI-2), and T-cell dependent (TD) antigen

Based on the structure, antigens can induce different humoral responses. T-cell independent antigens are characterised either by their ability to bind to different receptors on the B–cell (TI-1), or to activate accessory cells that provide activation and survival signals to the effected B cells (TI-2). T-cell dependent (TD) antigens on the other hand require antigen processing and presentation, and the presence of antigen specific T helper cells.

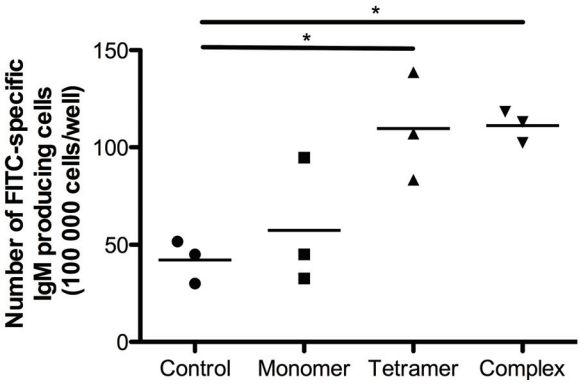
In order to test whether the b-2.4G2 scFv-avidin-FITC complexes have a similar effect on TD and TI-2 responses, antibody production of KLH-FITC (TD antigen) immunised mice was also studied. A set of different controls including monomeric 2.4G2 scFv and b-2.4G2 scFv coupled to avidin (tetramer b-2.4G2 scFv) was also involved, to see whether the aggregation in the absence of the antigen are contributing to the elevated humoral immune response mediated by the complex.

Mice received the preformed complexes on day 3 after FITC-dextran immunisation and on day 3 and day 6 after KLH-FITC immunisation. FITC specific IgM and IgG production was monitored on day 6 and day 9 in FITC-dextran and in KLH-FITC immunised mice, respectively.

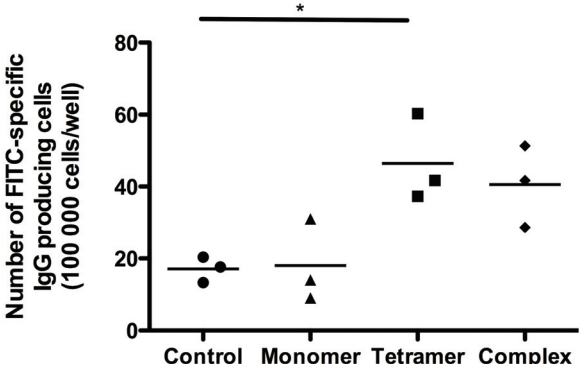
As shown in Figure 13., the complex caused a significant elevation in the number of FITC-specific antibody forming cells both in case of TI-2 and TD antigens. Moreover, tetramer biotinylated 2.4G2 scFv-avidin had a similar enhancing effect on the TI-2 but not on the TD immune response. KLH-FITC was injected as an aqueous suspension of Freund's adjuvant, causing not only the antigen specific activation of immune cells but also inducing inflammation, so the possible cytokine release exerted by the tetramer had no additional effect on the TD-response.

We could conclude that the monomeric 2.4G2 scFv is not capable to induce any changes in the number of FITC-specific IgM/IgG producing cells, but the aggregation of the protein is enough to mediate increased antibody levels in response to the TI-2 antigen FITC-dextran. The complex induces elevated antibody responses in both TI-2 and TD-antigen immunsed animals.

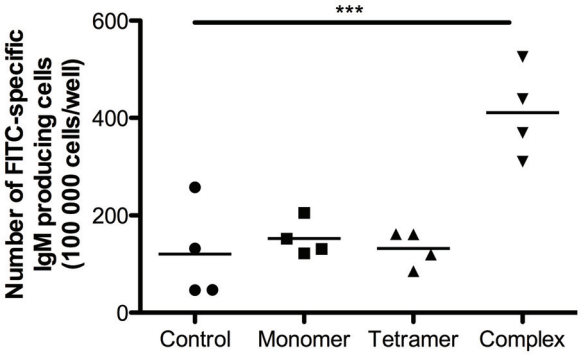
(a)


*
*
150
100
50
0
Number of FITC-specific IgM producing cells (100 000 cells/well)
Control
Monomer
Tetramer
Complex

(b)


*
80
60
40
20
0
Number of FITC-specific IgG producing cells (100 000 cells/well)
Control
Monomer
Tetramer
Complex

(c)



(d)

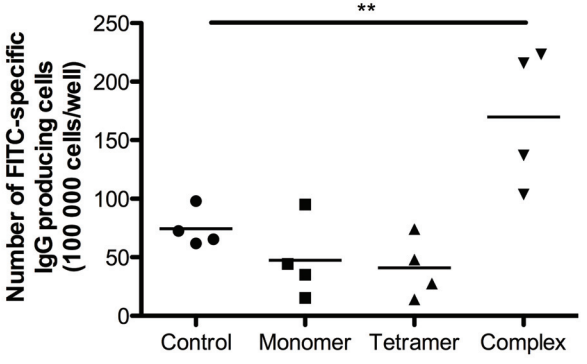
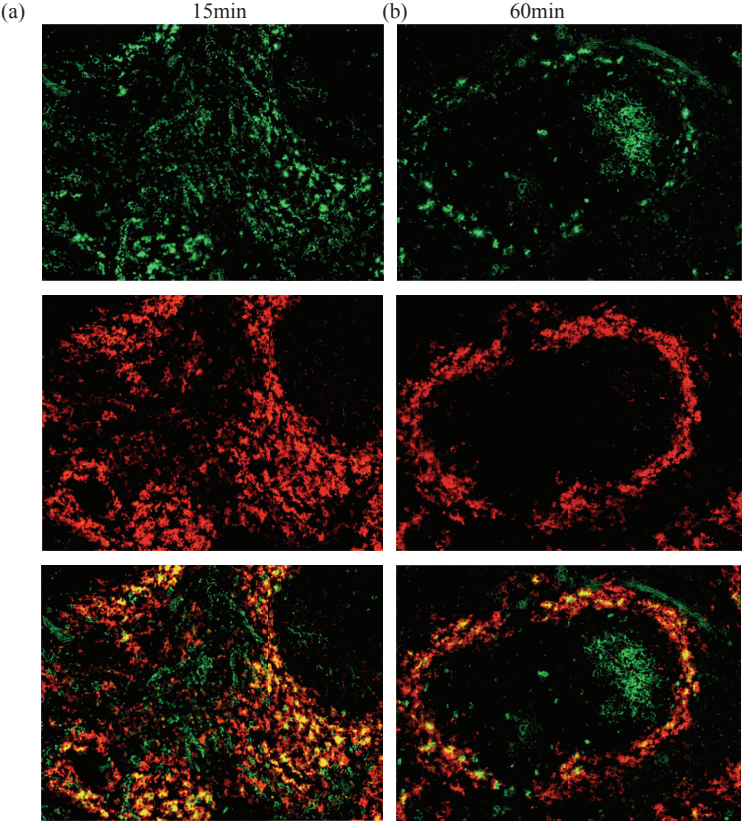


Figure 13.: Comparison of the effect of the 2.4G2 scFv on the T cell dependent (TD) and T cell independent 2 (TI-2) response of B cells. Mice were immunised with FITC-dextran (TI-2 antigen) or KLH-FITC (TD antigen), then treated with monomeric or tetrameric 2.4G2 scFv or the complex and FITC-specific IgM and IgG producing B cells were detected in an ELISPOT assay 3 days after last booster treatment. Number of FITC-specific IgM and IgG producing cells was examined both in the FITC-dextran immunised (Figure 13.a and b) and KLH-FITC immunised (Figure 13.c and d), tetramer or complex treated groups.

4.10 Localisation of the complexes in the spleen following TI-2 and TD immunisation

Next we immunised animals intraperitoneally with a suboptimal dose of FITC-dextran (Figure 15.a and b) or KLH-FITC (Figure 15.c and d), and injected them with the preformed complexes intravenously on day 3. The signal of the 2.4G2 scFv-avidin-FITC complex was detected by fluorescent microscopy. Co-staining with MARCO (red) showed that in immunised animals the complex has a localisation similar to that in naïve animals: after 15 minutes it is already present on marginal zone macrophages, and by 60 minutes it is partially internalised, and cleared out from the red pulp. Additionally, in TI-2 immunised animals we could observe the presence of the complex on the follicular dendritic cell network



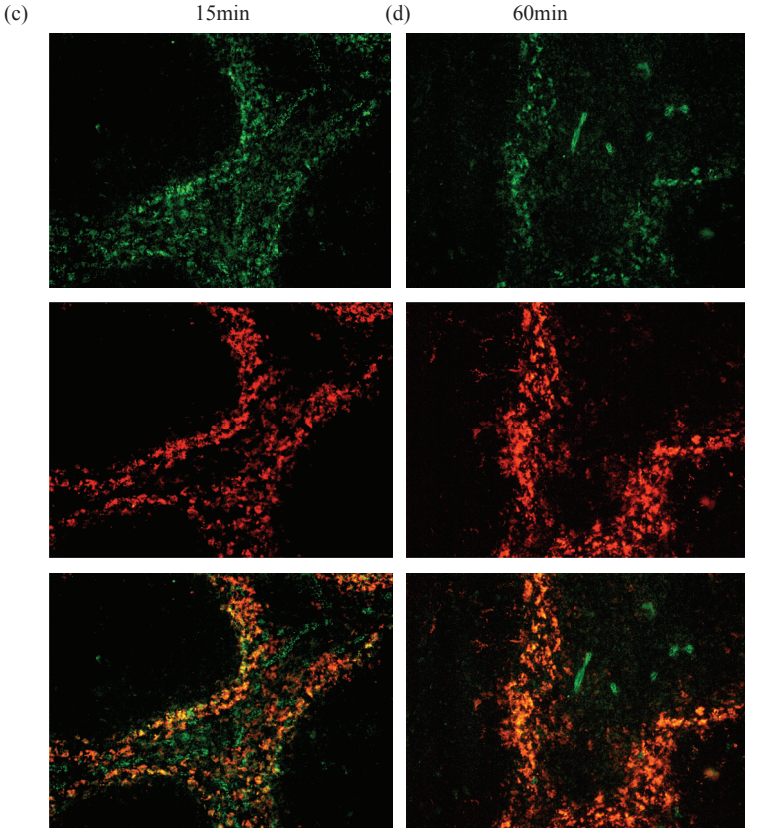


Figure 14.: FITC-dextran (a and b) and KLH-FITC (c and d) immunised animals (n=2/group) were treated with the b-2.4G2 scFv-avidin-FITC complex, and spleens were removed 15 or 60 minutes after i.v. injection.

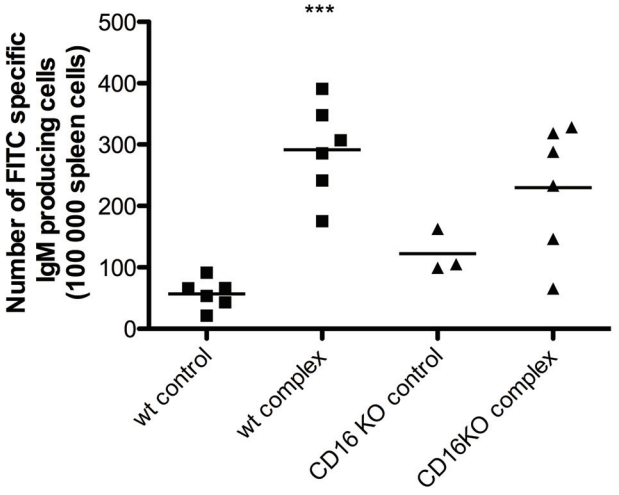
4.11 Elevation of the number of antibody producing cells after treatment with the b-2.4G2 scFv-avidin-FITC complex requires CD16 (FcγRIII) both in case of TI-2 and TD antigen immunised mice

The monoclonal antibody 2.4G2 recognises a determinant expressed on both FcγRIII (CD16) and FcγRII (CD32) molecules, raising the question whether the single receptor CD16 knock out mice would respond to the b-2.4G2 scFv-avidin-FITC complex treatment similarly to their wild type littermates. Therefore, CD16KO C57BL/6 mice were also immunised with TI-2 or TD antigens then treated with intravenously administered b-2.4G2 scFv-avidin-FITC, similarly to the wild type counterparts.

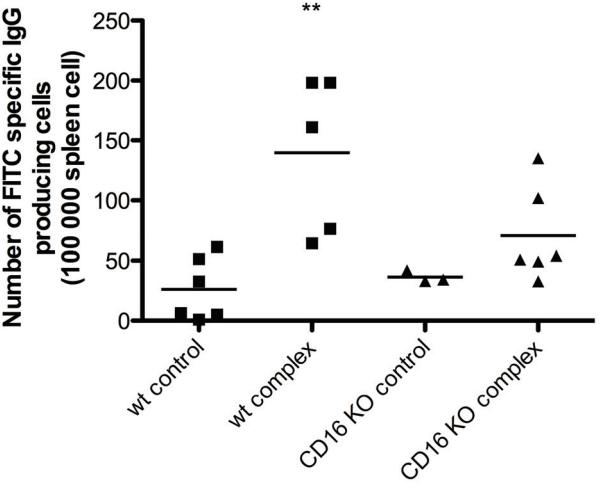
The lack of CD16 expression caused an enhancement in the IgM response towards FITC-dextran that could not be further modified by the complex-treatment (Figure 15.a). Interestingly though, the IgG-response of these animals towards the antigen was not different from the wild type mice, but could not be triggered by the injected complex (Figure 15.b).
The hapten specific IgM and IgG response in KLH-FITC immunised mice following complex treatment was significantly reduced compared to the wild type response (Fig.15c and d).

Taken together, our data suggest that CD16 (FcγRIII), the activating type receptor is mainly responsible for the enhanced antibody synthesis under these experimental conditions.

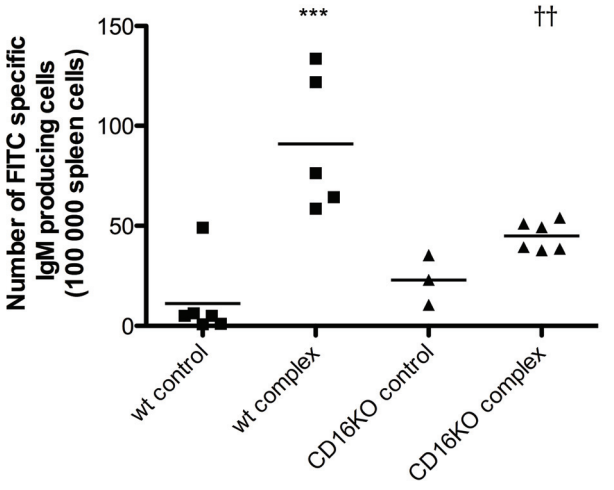
(a)


Number of FITC specific IgM producing cells (100 000 spleen cells)
500
400
300
200
100
0
***
wt control
wt complex
CD16 KO control
CD16KO complex

(b)


Number of FITC specific IgG producing cells (100 000 spleen cell)
250
200
150
100
50
0
**
wt control
wt complex
CD16 KO control
CD16 KO complex

(c)



(d)

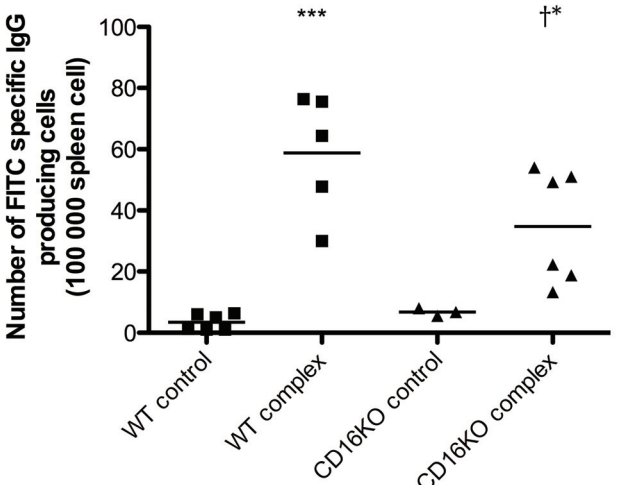


Figure 15.: C57BL/6 wt and CD16KO mice were immunised with TI-2 or TD antigens, and the number of FITC specific IgM/IgG producing cells was detected in an ELISPOT assay. Data provided by Dr. J. Prechl and Zs. Szekeres.
*: $p<0.05$, **: $p<0.01$, ***: $p<0.005$ control vs. complex, †: $p<0.05$, ††: $p<0.01$ wt vs. CD16KO)

5. Discussion

Fc-receptors binding monomeric immunoglobulin or ICs have a crucial role in the initiation and regulation of normal and pathological immune response. Although this family of proteins was described 40 years ago, data about their *in vivo* function only emerged in the last decade, mainly based on studies carried out in knock-out animals.

Antigen presenting cells express a range of FcγRs, therefore the lack of expression of any of these activating or inhibitory receptors has robust effects on the immune response. FcγRI-deficient mice showed a wide spectrum of defects in Fc-dependent effector cell function: reduced endocytosis of IgG, impaired phagocytosis of ICs, defected ADCC reactions, failures in the delivery of ICs to antigen presentation pathways, and interestingly, elevated antibody responses (47) (20). FcγRIIB knock-out mice spontaneously develop a lupus-like disease (48) (49) and had an elevated level of auto-antibodies, while autoimmune prone mouse strains express reduced levels of CD32 on activated and GC-B cells. FcγRIIB exerts its regulatory function during the late stages of B-cell maturation, preventing the expansion of autoreactive B-lymphocytes and their development into plasma cells. In collagen-induced arthritis (CIA), a mouse model of rheumatoid arthritis, FcγRIII was critically required in early disease onset, while destructive arthritis was developed in the absence of the activating Fc-receptors CD64/16, most probably due to the activity of FcγRIV (13). Fcα/μR, expressed mainly on marginal zone B cells, follicular B cells and FDCs, was reported to be a negative regulator of humoral immune responses against TI-antigens (50).

During the course of a humoral immune response ICs may engage different types of FcγRs, inducing a complex series of events. Administration of IgG containing ICs *in vivo* may inhibit or enhance antibody production depending on whether activating and/or inhibitory FcγRs are involved in the processes (4). Co-expression of both receptors on the same cell may set a threshold for cell activation, while shifting the balanced signalling may lead to the development of excessive activation, chronic inflammation or even an autoimmune disease (11, 12). To clarify these divergent effects, we aimed to investigate the regulatory function of artificial ICs composed of FcγRII/FcγRIII specific biotinylated 2.4G2 scFv and avidin-FITC on the FITC-specific humoral immune response.

In our experiments we intended to apply ICs of defined composition, therefore we produced a mono-biotinylated mouse FcγRII/III specific scFv construct from the monoclonal antibody 2.4G2 that was reported to recognise both FcγRII and FcγRIII, but does not bind to FcγRI or

FcγRIV (51), and coupled it to FITC-labelled avidin in a molar ratio of 4:1. FITC as a hapten conjugated either to dextran or to KLH was used to trigger T-independent type 2 or T-dependent immune response in mice. FITC has several advantages: the dye can usually be detected even after several hours of recirculation if injected to animals, furthermore, depending on the carrier molecule, it provides a possibility to study the effect of the FITC containing ICs either on TI-2 or TD responses specific for the same hapten. Thus we used biotin-2.4G2 scFv-avidin-FITC, as a model for ICs that may bind simultaneously both to the inhibitory and to the activating FcγRs and examined their regulatory role upon TI-2 and TD responses.

First, we characterised the binding capacity of the monomeric scFv and the pre-formed complex to A20 cells and also to mouse splenocytes *in vitro*. Single chain antibody fragments usually show the same specificity as the whole antibody with a slightly reduced affinity towards the antigen epitope they recognise. The assays showed that the antigen recognition ability of 2.4G2 scFv was intact, and comparable to the original antibody, moreover, the scFv was able to compete with the whole antibody for epitope-binding.

The *in vivo* experiments demonstrated that 15 and 60 minutes after intravenous injection the 2.4G2 scFv-avidin FITC complex bound to a fraction of B220+, CD11b+ and CD11c+ splenocytes, corresponding to a small fraction of B cells, macrophages and dendritic cells, respectively, as shown by flow cytometry. However, unlike the mouse CR1/2 specific 7G6 scFv (52), the 2.4G2 scFv containing complexes were filtered out from the circulation and degraded within 3 hours. The kinetics of the b-2.4G2 scFv–avidin-FITC treatment has shown that it was efficient if added 3 days after the initial immunisation with TI-2 antigen, and since we have found that the complex has degraded within 3 hours, these data suggest that the 2.4G2 scFv complex may exert its effect in the central phase of the immune response, in course of the development of antibody forming cells.

Experiments focusing on the delivery of blood-borne pathogens to splenic follicles showed that marginal zone B (MZB) cells rapidly capture opsonised antigens e.g. Ficoll via CR1/2, and that these cells are dislocated from the marginal zone following LPS or pertussis toxin treatment. Others reported that despite of heavy loading of TNP-Ficoll on MZB cells 30 minutes after injection, the antigen is cleared of surface by 2-3 hours, a process that seems to depend on cellular shuttling between the marginal zone and the follicle. Due to the very high expression of CR1/2 on the cell surface, FDCs are then thought to successfully compete for binding of opsonised antigens in the follicules (53).

In order track the localisation of our complexes within the spleen, we used immunofluorescent staining of frozen sections. The i.v injected b-2.4G2 scFv-avidin-FITC localised first on sinusoid endothelial cells then on marginal zone macrophages, and were internalised 60 minutes post injection. By the third hour of observation, neither the FITC signal nor the c-myc tag on 2.4G2 scFv could be detected in the spleen. We observed a co-localisation of FITC signals with MARCO, a marker for marginal zone macrophages. These data suggest that the circulating model immune complexes first bind to the FcγRs on sinusoid endothelial cells (54), and then to marginal zone macrophages, whose main function is to take up blood borne antigens (55). Marginal zone macrophages express the murine homologue of the lectin binding receptor, DC-SIGN, which was recently shown to bind 2,6 sialylated IgG (56). The immuncomplexes composed of 2.4G2 scFv and avidin do not contain sialic acid, thus bind to FcγRII and FcγRIII but not to the DC-SIGN homologue SIGN-R1 molecules, therefore, the uptake of the model complexes seems to be driven by FcγRII/III-mediated endocytosis .

Despite of the relatively high expression of FcγRII on marginal zone B cells (57), the complex seemed not to stain the marginal zone B cells nor get through to the follicles within the white pulp of the spleen, probably due to the activity of marginal zone macrophages. The discrepancies between the data measured by flow cytometry and immunohistochemistry could originate from differences in the sensitivity of the two detection methods.

Localisation of i.v injected b-2.4G2-avidin FITC immune complexes was also studied in mice previously immunised with KLH-FITC or dextran-FITC, where we could detect a similar binding pattern, namely a co-localisation of the complexes with marginal zone macrophages (MARCO+ cells) and with sinusoid endothel cells. In addition, the binding of b-2.4G2 scFv-avidin-FITC to FDC reticula was also observed in case of the TI-2 response. Rao et al. reported that FcγRIIb mRNA and protein levels in GC B cells are down-regulated, as compared to non-GC B cells, which indicates that modulation of the B cell response within the GCs might not occur through this inhibitory receptor (57). On the other hand, Rao et al also found that FcγRIIb was up-regulated on FDC, during the germinal centre response. In concert with these findings our data show that the *in vivo* administered 2.4G2 scFv-avidin-FITC complexes cannot be detected on B cells, consequently do not regulate through FcγRIIb1, but are trapped in the macrophages in the marginal zone and on FDCs, thus may regulate via FcγRIIb2 and/or via FcγRIII expressed on these cells.

The targeted transport of the antigen to the marginal zone macrophages and FDCs resulted in an elevated FITC-specific humoral response 6 days after primary immunisation with TI-2

antigen. As the scFv had been produced in a bacterial protein expression system, we had to rule out the possibility that the detected effect is mediated by bacterial lipopolysaccharid (LPS). Neither addition of LPS nor treatment with the LPS neutralizing agent, polymixin B modified the level of antibody synthesis, indicating that the enhanced IgM and IgG secretion in response to TI-2 antigen induced by the 2.4G2 scFv-avidin-FITC highly depends on FcγRII/III. Non-biotinylated, monomeric 2.4G2 scFv molecules in the presence of avidin-FITC, did not modify antibody synthesis. However, 2.4G2 scFv-tetramers could prime the B cell response in an extent comparable to the complex, but only in case of a TI-2 antigen. These data indicate that cross-linking of FcγRII/III, thus inducing FcγRs-mediated activating signals is indispensable for the effect. Cell activation via FcγRIII may mediate the release of pro-inflammatory cytokines (58), thus we suggest that the enhanced antibody synthesis in case of TI-2 antigen and the appearance of IgG isotype could be a result of FcγRIII-induced cytokine production, e.g. IL-6, TNFα or IFNγ (Figure 16.). In a recent publication, Rittirsch et al. report on IC-mediated IL-6- and TNFα–release in a mouse model of acute lung injury, where they demonstrate that the association of TLR4 is essential to proper FcγRIII functions, namely to the phosphorylation of the signal-transducing γ-chain. Our further studies will involve local detection of inflammatory cytokine release within the spleen following complex-treatment.

In contrast to the TI-2 response, tetramer scFv 2.4G2 did not modify the B cell response to TD antigen. In course of immunisation with the TD antigen KLH-FITC in complete Freund's adjuvant, an appropriate level of cytokines might be synthesised (59) (60), (61), thus FcγRII/III cross-linking by the tetramer scFv 2.4G2 has no additional cytokine mediated effect. However, the model complex, b-2.4G2 scFv-avidin-FITC significantly enhanced both IgM and IgG production to TD antigen as well compared to the untreated control. This could be the result of the formation of larger complexes by binding circulating FITC-specific IgG antibodies to avidin-FITC, such a process may enhance antigen presentation and ultimately antibody formation.

The monoclonal antibody 2.4G2 recognises both the activating CD16 and the inhibitory CD32 receptors, as these proteins show about 90 % sequence homology in their extracellular parts in mice. Previous reports have shown that immune complex induced enhancement of bacterial antigen presentation requires expression of FcγRIII on DCs (62). Moreover, it was reported that immune complex-loaded DCs can prime a T independent response in an FcγRIIb-dependent manner (63). In order to decide which receptor is responsible for the enhanced antibody synthesis, we immunised CD16KO animals with both TI-2 and TD antigen,

respectively, and compared their antibody production and its regulation by 2.4G2 scFv-avidin-FITC complexes with the wild type littermates. CD16KO mice immunised with TI-2 antigen, dextran-FITC had a slightly higher number of hapten specific IgM producing cells in the spleen, but the booster injection with 2.4G2 scFv-FITC complexes did not induce a significant elevation of the response. We could not detect significant differences in the number of FITC specific IgG producing cells either with or without the i.v. injection of the 2.4G2 scFv complexes, indicating that the FcγRIII is indispensable for the effect.

The antibody response to the TD antigen KLH-FITC was also highly significantly enhanced by 2.4G2 scFv-avidin FITC complexes in case of wild type animals, which was less pronounced in CD16KO mice, indicating that FcγRIII may only partly be responsible for the immune complex-induced enhancement of antibody synthesis upon TD response.

Our results demonstrate that ICs, which are able to bind to both FcγRII and FcγRIII, preferentially bind to the later *in vivo*, eventually resulting in an enhanced antibody response.

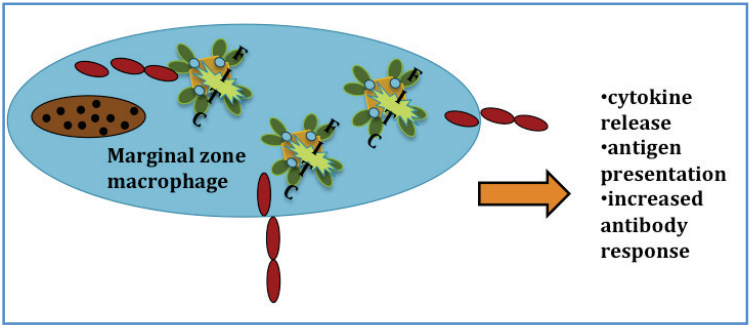


Figure 16.: Schematic model of the possible act of mechanism

Taken together these results suggest that immune complexes predominantly act *in vivo* as positive regulators of antibody production, which may be mediated by the induction of cytokine synthesis via FcγRIII cross-linking. Immune complexes bound to FcγRII/FcγRIII on marginal zone macrophages and dendritic cells may also promote antigen presentation to T cells or B cells, thus enhancing antibody synthesis (64). These data also indicate that FcγRIII are potential drug target in various immune-mediated diseases.

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Summary.

Fcγ-receptors play an important role in both the afferent and the efferent phase of the immune response: while the antigen uptake and presentation is highly facilitated by the activating-type Fcγ-receptors on antigen presenting cells, the inhibitory FcγRIIb is an essential mediator of the down-regulation of B cell activation in the late phase.

In the present work we aimed to further examine the effect of immune complexes on the humoral response. Single chain Fragment variables (scFv) represent a widely applied tool for cellular targeting as they lack the Fc-part that mediates effector functions. Therefore we designed an scFv from the well-characterised clone 2.4G2, specific for the mouse CD16/CD32 (FcγRII/FcγRIII). We amplified and isolated the heavy and light chain variable regions, cloned them into an expression vector, and in a set of experiments we showed that the recombinant protein is intact and recognises the same epitope as the whole antibody.

For the *in vivo* studies we prepared a complex of b-2.4G2 scFv-and avidin-FITC in a ratio 4:1 and injected the complex to naïve C57/BL6 mice to check the localisation of the complex in the spleen in various time points. The complex was mainly localised on red pulp endothelial cells (IBL $9/2^{+}$ cells) by 15 minutes, but got translocated to the $MARCO^{+}$ marginal zone macrophages by 60 minutes, and was completely cleared out of the circulation after 3 hours.

To characterise the effects of the 2.4G2 scFv on the humoral response, we immunised animals with the TI-2 antigen FITC-dextran or the TD antigen KLH-FITC, and injected the mice with the complex intravenously as a booster. In ELISPOT assays we analysed the FITC-specific antibody responses on day 6 (TI-2 antigen) or day 9 (TD-antigen), and detected a significant elevation in the number of IgM and IgG producing cells in the spleen. As the clone 2.4G2 recognises both the mouse CD16/32, we immunised a group of CD16KO animals to decide whether the lack of FcγRIII has any influence on the complex-mediated changes. We found a somewhat elevated response to the TI-2 antigen in CD16KO animals that was not altered by the complex significantly, while the animals immunised with TD antigen showed a significantly reduced response to the complex compared to their wild type littermates.

Taken together these data indicate that immune complexes predominantly act *in vivo* as positive regulators of antibody production and CD16 is largely responsible for this effect. Furthermore, these data suggest that FcγRIII is a potential drug target in various immune-mediated diseases.

Összegzés

Az Fcγ-receptorok nagyon fontos szerepet játszanak az immunválaszban, csakúgy az afferens, mind az efferens szakaszban: míg az antigén prezentáló sejteken expresszált aktiváló Fcγ-receptorok nagyban elősegítik az antigén-felvételt és prezentációt, a gátló típusú FcγRIIb a B-sejt aktiváció gátlásának nagyon fontos közvetítője a késői fázisban.

Az itt ismertetett munkával az volt a célunk, hogy megvizsgáljuk az immunkomplexek hatását a humorális immunválaszra. Az egyláncú ellenanyagokat (scFv) széles körben használják különböző komplexek adott sejtek való irányítására, mivel nem rendelkeznek az effektor funkciókat kiváltó Fc-fragmentummal. Kísérleteink során a jól jellemzett egér CD16/32 specifikus 2.4G2 klónból egylácú Fv fragmentumot hoztunk létre. Felszaporítottuk és izoláltuk a nehéz, és könnyű lánc variábilis régiókat, majd ezeket expressziós vektorba klónoztuk be, amelynek segítségével scFv fehérjét termeltünk. Kísérleteinkben megmutattuk, hogy a rekombináns fehérje intakt, és ugyanazt az epitópot ismeri fel az FcγRII/FcγRIII-on, mint a teljes 2.4G2 ellenanyag.

Az *in vivo* kísérletekhez a b-2.4G2 scFv és avidin-FITC 4:1 arányú komplexét oltottuk naív C57/BL6-os egerekbe, és ellenőriztük a komplexek lokalizációját a lépben különböző időpontokban. 15 perccel az oltás után a komplexek főleg a vörös pulpa endothel sejtjeinek felszínéhez kötődtek (IBL $9/2^+$ sejtekhez), 60 perc múlva áthelyeződtek a $MARCO^+$ marginális zóna makrofágokra, míg 3 óra múlva teljesen kiürült a keringésből.

A 2.4G2 scFv humorális válaszra kiváltott hatásának jellemzése érdekében FITC-dextránnal (TI-2 antigén), vagy KLH-FITC-cel (TD antigén) immunizált állatokat kezeltük a komplexszekkel. ELISPOT módszerrel elemezve a FITC-specifikus ellenanyag választ, a 6. (TI-2 antigén) vagy a 9. (TD antigén) napon szignifikáns növekedést tapasztaltunk az IgM és IgG termelő lépsejtek számában. Mivel az ellenanyag mindkét receptort (CD16/32) felismeri, CD16 génkiütött (KO) egereket is immunizáltunk annak eldöntésére, hogy receptor hiánya hatással van-e a komplex-mediált változásokra. A TI-2 antigénnel kezelt állatok esetében a CD16KO állatoknál kissé megemelkedett ellenanyag-választ tapasztaltunk, amelyet nem befolyásolt a komplexszel történő kezelés, míg a TD antigénnel immunizált CD16KO egerek komplex-kezelésre adott válasza szignifikánsan alacsonyabb volt a vadtípusú csoporténál.

Eredményeink azt jelzik, hogy az főként az FcγRIII tehető felelőssé a komplexek által kiváltott immunválasz serkentő hatásért.

Összegezve, adataink azt sugallják, hogy az immunkomplexek túlnyomóan pozitív hatással vannak az ellenanyagtermelésre *in vivo*, és hatásukat az FcγRIII (CD16) közvetíti. Így az FcgRIII terápiás célpontul szolgálhat számos immunrendszeri betegség kezelése során.

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